Release of Prostaglandins from the Uterus

by

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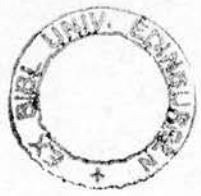

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SUMMARY

There is much evidence for the existence of a luteolytic hormone (luteolysin) secreted by the uterus towards the end of the oestrous cycle or pseudopregnancy in several mammalian species. Its chemical identity is unknown. Distension of the uterus by the insertion of a foreign body or the systemic administration of oestrogen causes early regression of the corpora lutea due to the premature release of this hormone.

Prostaglandin $F_{2\alpha}$ possesses potent luteolytic activity in all of the species so far tested. Consequently experiments have been performed to investigate whether the uterine luteolytic hormone could be this prostaglandin.

Distension of the guinea-pig uterus *in vitro* and the systemic administration of oestrogen to guinea-pigs were found to release prostaglandin $F_{2\alpha}$ from the uterus. The analysis of uterine venous blood samples taken from cycling sheep and guinea-pigs showed prostaglandin $F_{2\alpha}$ to be present in levels higher at the end of the oestrous cycle than at times earlier. Also the uterine venous blood of guinea-pigs contained a high level of prostaglandin E_2 at the end of the cycle.

In sheep with an ovary autotransplanted to the neck, cyclic activity ceases. The fluid which often accumulates in the uterus of such sheep has been analysed and was found to contain large amounts of prostaglandin $F_{2\alpha}$. In addition, prostaglandin F-like activity was detected in the uterine venous blood but not the carotid arterial blood of one of these sheep. The results of these findings are discussed.

Prostaglandins $F_{2\alpha}$ and E_2 were present in guinea-pig uterine tissue in small amounts at the end of the oestrous cycle. Guinea-pig uteri taken on selected days throughout the cycle were found capable of biosynthesising prostaglandins $F_{2\alpha}$ and E_2 from endogenous precursors during incubation *in vitro*. On any one day, 4 to 5 times

more prostaglandin $F_{2\alpha}$ than E_2 was produced, with greater amounts of each being formed nearer to the end of the oestrous cycle. Indomethacin inhibited this synthesis of prostaglandins.

The results obtained in this work support the hypothesis that the uterine luteolytic hormone (luteolysin) is prostaglandin $F_{2\alpha}$. This view is discussed in relation to connected findings and observations reported by other workers.

CONTENTS

INTRODUCTION

In 1923, Loeb observed that total hysterectomy prolonged the life span of the corpora lutea in guinea-pigs. Oestrous cycle length was extended from the normal 17 days to beyond 60 days. Subsequently it was shown that hysterectomy performed in the cycling sheep, cow (Wiltbank and Casida, 1956) and pig (Spies, Zimmerman, Self and Casida, 1960) or in the pseudopregnant rat (Bradbury, 1937), rabbit (Asdell and Hammond, 1933) and hamster (Caldwell, Mazer and Wright, 1967) prevented the normal regression of corpora lutea, also. However, hysterectomy performed in the dog (Cheval, 1934), squirrel (Drips, 1919), ferret (Deanesly and Parks, 1933), opussum (Hartman, 1925), monkey (Burford and Diddle, 1936), human (Jones and Te Linde, 1941) and in the marsupial Trichorus vulpecula (Clark and Sharman, 1965) was without effect on corpus luteum life-span. It is therefore apparent, that in several mammalian species the uterus exerts some control over corpus luteum function, regulating the length of the oestrous cycle or of pseudopregnancy.

In the hemi-hysterectomised guinea-pig (Fischer, 1965) and sheep (Inskeep and Butcher, 1966; Moor and Rowson, 1966a) regression of corpora lutea occurred only in the ovary adjacent to the retained horn. In the pig, however, hemi-hysterectomy resulted in only slightly extended oestrous cycles (Anderson, Butcher and Melampey, 1961). If less than one quarter of one horn remained intact though, regression of corpora lutea occurred only in the ovary adjacent to the retained portion of horn (du Mesnil du Buisson, 1961). In the unilaterally pregnant pig, corpora lutea in the ovary adjacent to the non-gravid horn regressed as normal, whilst corpora lutea in the opposite ovary were retained (Anderson, Rathmaker and Melampey, 1966). This phenomenon was also seen in the guinea-pig (Oxenreider and Day, 1967). In the hamster (Duby, McDaniel, Spilman and Black, 1969a) and rat (Anderson, Melampey and Chen,

1966), the duration of pseudopregnancy was significantly lengthened by the removal of one uterine horn, or one horn and the contralateral ovary. When the ovary and the horn were removed from the same side, normal luteal regression occurred. All these observations are indicative of a direct local relationship between an ovary and its adjacent uterine horn, without the involvement of the pituitary as mediator.

Autotransplantation of the uterus in the hamster (Caldwell et al, 1967) or of the ovary in the rat (Anderson et al, 1966a) resulted in a lengthened pseudopregnancy. In the pig (Spies, Zimmerman, Self and Casida, 1960) and guinea-pig (Loeb, 1927) uterine autotransplantation had the same effect as total hysterectomy, although subsequent workers have reported only slightly extended cycles following this procedure in both these species (Anderson et al, 1961; Butcher, Chu and Melampey, 1962a). In sheep, autotransplantation of the uterus or ovary to the neck prevented corpus luteal regression (Goding, Harrison, Heap and Linzell, 1967a; Goding, McCracken and Baird, 1967b). However, if the ovary and uterus together were transplanted to the neck, regular oestrous cycles were maintained (Harrison, Heap and Linzell, 1968). These observations indicate that the controlling influence exerted by the uterus on the ovary is disturbed by a change in anatomical siting that involves separation of the two organs. Furthermore, the nervous system cannot be involved since autotransplantation of the two organs together had no adverse effect. This is substantiated by the fact that uterine denervation in the pig had no effect on oestrous cycle length (Anderson and Melampey, 1962a).

It has therefore been postulated that in some mammalian species a luteolytic hormone (luteolysin) is released from the uterus which acts on the ovaries in a local rather than in a systemic manner. This hypothesis was one of several possible mechanisms proposed by Loeb as far back as 1927. The apparent source of this hormone is the endometrium for endometrial destruction in the guinea-pig had the same effect as total hysterectomy (Butcher, Chu and Melampey, 1962b). The pathway for the hormone appears to be provided

by the vascular system or lymph vessels. Severance of the vascular tissue between the uterus and ovary in guinea-pigs caused maintenance of the corpora lutea, whereas the severance and removal of part of the fallopian tube had no effect on corpora luteal regression (Oxenreider and Day, 1967; Bland and Donovan, 1969a). In the rat, ligation and severing of the oviducts and the accompanying utero-ovarian vessels in unilaterally ovariectomised-hysterectomised or intact animals extended pseudopregnancy (Butcher, Barley and Inskeep, 1969), whereas interruption of the oviduct alone did not have this effect (Barley, Butcher and Inskeep, 1966). In the sheep, bilateral ligation of the middle uterine arteries and veins prevented regression of the corpora lutea (Kiracofe, Spies and Gier, 1963; Kiracofe, Menzies, Gier and Spies, 1966). Unilateral ligation of the same vessels ipsilateral to the ovary containing the corpus luteum had a similar effect, whereas ligation of these vessels on the contralateral side to the ovary containing the corpus luteum had no effect on oestrous cycle length. In addition, bilateral or unilateral ligation of the middle uterine arteries alone did not alter the length of the oestrous cycle. These observations are in keeping with the local nature of the utero-ovarian relationship and indicate that the venous drainage from the uterus is involved.

Regression of the corpora lutea can be induced to occur prematurely under certain conditions. Distension of the uterus on days 2 - 4 of the cycle by the insertion of a foreign body into the uterine horns of the guinea-pig (Donovan and Traczyk, 1962), sheep, (Moore and Nalbandov, 1953) and cow, (Hansel and Wagner, 1960) caused shortened oestrous cycles due to the early regression of the corpora lutea. If the foreign body was placed in only one uterine horn, early regression of the corpora lutea in the ipsilateral ovary occurred whereas corpora lutea in the contralateral ovary regressed at the normal time in all three species (Bland and Donovan 1965; Ginther, Woody, Janakiraman and Casida, 1966). It appears therefore that the distension of the uterus

leads to the premature release of the luteolytic hormone independently from each horn. In the pig, however, uterine distension had no effect on oestrous cycle length (Anderson, 1962). In the sheep denervation of the distended segment of uterine horn prevented the expected early regression of the corpus luteum (Moore and Malbandov, 1953). The technique used though, involved severance of the blood vessels to and from that segment of horn. Since the vasculature provides the pathway for the luteolytic hormone, the result obtained was probably due to the severance of the blood vessels rather than to the severance of nerves.

Certain hormonal treatments can cause early regression of the corpora lutea also. Oestrogen administration during the early- or mid-part of the cycle in the guinea-pig (Bland and Donovan, 1968a; Choudary and Greenwald, 1968), sheep (Stormshak, Kelley and Hawk, 1968, 1969) and cow (Greenstein, Murray and Foley, 1958) caused shortened oestrous cycles due to early regression of the corpora lutea. Similar treatment in the pseudopregnant or pregnant hamster (Greenwald, 1965; Choudary and Greenwald 1969a) and in the pregnant pig (Rigor, Self and Casida, 1963; Gardner, First and Casida, 1963) significantly reduced corpora luteal weight at the time of autopsy, although in the pseudopregnant or pregnant rabbit oestrogen treatment had a luteotrophic effect (Hammond and Robson, 1951). Follicle stimulating hormone (FSH) administration can counteract, in part, the luteolytic effect of oestrogen in the guinea-pig (Choudary and Greenwald, 1969b) and cow (Wiltbank, Ingalls and Rowden, 1961), whilst in the hamster FSH abolished completely the luteolytic effect of oestrogen (Choudary and Greenwald, 1969a). In the guinea-pig, luteinizing hormone (LH), prolactin or a combination of both did not reverse the effect of oestrogen treatment (Choudary and Greenwald, 1969b). Prolactin (LtH) was also without effect in the oestrogen treated, pseudopregnant hamster (Choudary and Greenwald, 1969a). However in the cow, LH treatment following FSH administration did reverse the effect of oestrogen completely (Wiltbank, et al,

1961). In the sheep, oestrogen administered on day 5 decreased the level of pituitary LH below control values when measured on day 7 (Howland, Kirkpatrick, Woody, Pope and Casida, 1968). These observations indicate that the pituitary may be involved in the early regression of corpora lutea produced by oestrogen administration. However, hysterectomy performed in the guinea-pig (Rowlands, 1962; Bland and Donovan, 1970) and sheep (Stormshak, et al, 1968, 1969) abolished completely the luteolytic effect of oestrogen. In the hysterectomised cow, oestrogen administration caused only partial corpora luteal regression (Kaltenbach, Niswender, Zimmerman and Wiltbank, 1964; Brunner, Donaldson and Hansel, 1969). Oestrogen treatment of hemi-hysterectomised guinea-pigs resulted in early regression of the corpora lutea only in the ovary adjacent to the retained horn. Corpora lutea in the opposite ovary were unaffected (Bland and Donovan, 1970). It is therefore apparent that the uterus is essential as a mediator for the luteolytic effect of oestrogen. The local nature of this effect is indicative of oestrogen treatment causing the premature release of the uterine luteolytic hormone from each horn independently.

Progesterone treatment when begun on day 1 of the cycle resulted in shortened oestrous cycles in the guinea-pig, sheep and cow, but not in the pig (Woody, First and Pope, 1967). This has again been shown to be due to the early regression of the corpora lutea (Ginther, 1969 ; Harms and Malven, 1969; Woody, Ginther and Pope, 1968) as indicated by corpora luteal size or weight. The luteolytic effect of progesterone was not present in the hysterectomised guinea-pig (Ginther, 1969) or sheep (Woody, et al, 1968). In the hemi-hysterectomised, hemi-ovariectomised guinea-pig (Ginther, 1969,) or in the hemi-hysterectomised sheep (Ginther, 1968) and cow (Woody, Ginther and Casida, 1967), progesterone treatment shortened oestrous cycle length only when the retained uterine horn was on the same side as the ovary containing the corpora lutea. Therefore, it appears that the progesterone, like oestrogen, causes

early regression of the corpora lutea due to the premature release of the uterine luteolytic hormone.

The same story seems to be true for oxytocin. Its administration to cows resulted in shortened oestrous cycles (Armstrong and Hansel, 1959). This was again due to early regression of the corpora lutea and could not be prevented by the simultaneous administration of LH or prolactin (Simmons and Hansel, 1964). Donaldson, Hansel and Van Vleck (1965) found that the oxytocin reduced the pituitary content of gonadotrophins and that in their experiments LH did in fact overcome early luteal regression induced by oxytocin. These observations indicate that the pituitary may be involved, although plasma levels of LH (the apparent luteotrophic hormone in cows - see Ginther, 1968) are unaffected by oxytocin treatment (Harms, Niswender and Malven, 1969). In unilaterally, hysterectomised cows oxytocin administration shortened oestrous cycle length only when the retained horn was on the same side as the ovary containing the corpus luteum (Ginther, Woody, Mahajan, Janakiraman and Casida, 1967). Total hysterectomy abolished completely the luteolytic effect of oxytocin (Armstrong and Hansel, 1959; Hansel and Wagner, 1960). Consequently the presence of the uterus is essential for early corpora luteal regression to occur. This indicates that the luteolytic effect of oxytocin, like that of oestrogen and progesterone, is mediated via the uterus. The premature release of the uterine luteolytic hormone would account satisfactorily for the effect observed. However, oxytocin did not alter luteal function in the pig (Duncan, Bowerman, Anderson, Hearn and Melampey, 1961), guinea-pig (Donovan, 1961), rabbit or rat (Brinkley and Nalbandov, 1963). Its effect appears to be specific to the cow.

Overwhelming evidence has accumulated, therefore, for the existence of a uterine luteolytic hormone (luteolysin) in some mammalian species. There have been many attempts to isolate and identify such a hormone but with only limited success.

Bradbury, Brown and Gray (1950) reported that rat endometrial suspensions hastened corpora luteal regression on reinjection into pseudopregnant, hysterectomised rats. However, ether-soluble or lyophilised homogenates of rat uteri were ineffective in shortening the length of pseudopregnancy in rats similarly hysterectomised (Kiracofe and Spies, 1966). Likewise aqueous or ether extracts of cow endometrium collected at different stages of the cycle were without effect in this preparation (Malven and Hansel, 1965). However, acetone dried powder preparations of late luteal and early oestral uteri from cows were found to be luteolytic in the majority of pseudopregnant rabbits tested (Williams, Johnston, Lauterbach and Fagan, 1967). Also, cow endometrial extracts obtained on days 14 and 16 of the cycle promoted luteolysis in pseudopregnant hamsters hysterectomised on day 7 (Duby, McDaniel, Spilman and Black, 1969b). Extracts from uteri on days 12 and 18 were ineffective. However it has been shown since that aqueous extracts of cow endometrium from days 10 to 13 of the cycle are capable of reducing luteal weight and progesterone content in the pseudopregnant, hysterectomised hamster (Lukaszewaska and Hansel, 1970). The luteolytic factor present was non-dialysable. Sephadex column chromatography and ammonium sulphate precipitation indicated the active fraction to be a protein of large molecular weight. Aqueous extracts of hamster uteri on days 6 or 7 of pseudopregnancy were ineffective in shortening the length of pseudopregnancy on re-injection into hysterectomised hamsters, also (Mazer and Wright, 1968). The luteolytic factor was thermolabile and non-dialysable. Similarly, aqueous extracts of sheep endometrium collected on days 14 or 15, but not on days 6 or 9, of the cycle were luteolytic when injected into the hysterectomised, pseudopregnant hamster (Caldwell, Moor and Lawson, 1969a). The luteolytic factor was again non-dialysable, but this time relatively heat stable. Sephadex column chromatography suggested the factor to be a molecular weight below 1500 (Caldwell, Moor, Rowson and Hay, 1969b). However, injections of ether soluble,

or lyophilised homogenates of sheep uteri failed to promote the regression of the corpus luteum in hysterectomised sheep (Kiracofe et al, 1963, 1966).

Endometrial filtrates from donor pigs on days 18 - 20 of the cycle infused into the endometrium of recipient pigs on days 12 or 13 failed to shorten oestrous cycle length (Anderson and Melampey, 1962b). However, luteolytic activity was detected in pig uterine flushings collected on days 14 - 18 but not on days 1 to 10 or 20 of the cycle (Schomberg, 1967). The luteolytic factor was thermolabile and non-dialysable. It appeared to be a high molecular weight protein. Similarly, an inhibition of the synthesis of progesterone, in vitro, was obtained by introducing into the incubation media endometrial extracts from pigs on days 16 to 18 of the cycle (Duncan et al, 1961). The luteolytic factor was dialysable and relatively heat stable. Extracts of the endometrium from day 12 or 13 pigs were found, surprisingly, to stimulate progesterone synthesis. Similar results have been obtained for pig endometrial slices taken on days 8 or 14 of the cycle (Stormshak and Kelly, 1967). Guinea-pig uterine extracts of unspecified time in the cycle were also able to inhibit the biosynthesis of progesterone, in vitro (Cooper and Hess, 1965). However Bland and Donovan found that aqueous, saline or acetone extracts from guinea-pig uteri did not influence corpora luteal size when injected into hysterectomised guinea-pigs (see Anderson, Bland and Melampey, 1969).

It is difficult to draw any conclusions about the identity of luteolysin from these observations since the overall picture is confusing and sometimes contradictory. However, the hormone appears to be present in the uterus towards the end of the oestrous cycle or pseudopregnancy, as would be expected, and to be protein-like in nature. It was found that LH caused the morphological regression of corpora lutea in rats bearing pituitary autografts (Rothchild, 1965). It was luteolytic also in pregnant and pseudo-pregnant hamsters when injected on day 1 through to day 5 (Choudary and Greenwald, 1969a).

Prolactin caused the morphological regression of corpora lutea also in rats when injected 48 hours after hypophysectomy. However, it is unlikely that these effects are truly physiological. It is equally unlikely that the uterus can produce these two pituitary hormones. In fact, some luteolytic extracts prepared from cow uteri were shown not to contain any LH-like substances by their inability to induce ovulation in rabbits (Williams et al, 1967). In hysterectomised guinea-pigs, several relaxin preparations were capable of inducing vaginal opening in half of the animals tested (Jagiello, 1967). Fresh ovulations had occurred in most of these guinea-pigs. However relaxin had this effect in ovariectomised guinea-pigs, also (Jagiello, 1965). Vaginal opening was, therefore, probably due to a direct effect of relaxin. However, the guinea-pigs uterus does appear to be capable of producing relaxin (Hisaw, Zarrow, Money, Talmage and Abramowitz, 1944). In the hysterectomised cow, relaxin injections did not cause regression of the corpus luteum (Brunner et al, 1969). Relaxin does not therefore appear to be luteolytic and it cannot really be considered as a candidate for the uterine luteolytic hormone, neither can LH nor prolactin. Consequently the identity of luteolysin despite all this detailed study still remains unsolved.

An interesting suggestion was put forward by Channing (see Anderson et al, 1969). He proposed that the discrepancies in the results so far obtained, could be explained by the fact that "the lytic factor may be bound to a protein". Several of the luteolytic extracts prepared from the uterus were found to contain small amounts of carbohydrate and/or lipid material (see Lukaszewaska and Hansel, 1970). These observations may become significant since one substance of low molecular weight, prostaglandin $F_{2\alpha}$, possesses potent luteolytic activity, <u>in vivo</u>. Experiments have shown it to be luteolytic in all the species so far tested, namely the rat (Pharriss and Wyngarden, 1969), rabbit (Gutknecht, Cornette and Pharriss, 1969), guinea-pig (Blatchley and Donovan, 1969) sheep (McCracken, Glew and Scaramuzzi, 1970), monkey (Kirton, Pharriss

and Forbes, 1970) and hamster (Labhsetwar, 1971). In vitro, however, prostaglandin $F_{2\alpha}$ was found to be luteotophic (Speroff and Ramwell, 1970).

Prostaglandin $F_{2\alpha}$ is one of the many naturally occurring prostaglandins. These substances are 20-carbon fatty acids containing a cyclopentane ring. They differ from one another in their degree of saturation and location of ketone and hydroxyl groups. They have been found to be present in many tissues and to be released from many more. Their pharmacological actions are numerous and they are usually extremely potent (see Horton, 1969).

Prostaglandins are formed from unsaturated, straight chain, fatty acid precursors, which in the case of prostaglandin E_2 and $F_{2\alpha}$ is arachidonic acid (see Bergström, 1966). The biosynthesis of these two compounds proceeds via a common intermediate (Samuelsson, Granström and Hamberg, 1967) (see Figure 1). The presence of certain co-factors, namely hydroquinone and reduced glutathione, probably decides the proportions of prostaglandin E_2 and $F_{2\alpha}$ formed by any one tissue (Nugteren, Beerthius and van Dorp, 1967).

The precise physiological role of prostaglandins still remains obscure. Prostaglandins of the "E" and "F" series (Ferriera and Vane, 1967), but not the "A" series (Horton and Jones, 1969), have been found to be efficiently removed from the circulation by one passage through the lungs. With the possible exception of those of the "A" series, it is improbable that prostaglandins act as circulating hormones. Their action may be local in nature, acting at the site of release or very close to it. They may modulate the actions of other substances in the body, in part by acting on the adenyl cyclase-cyclic AMP system (see Horton, 1969.) One or more of the prostaglandins have been found to be identical with darmstoff (Suzuki and Vogt, 1965), irin (Änggård and Samuelsson, 1964), the menstrual stimulants (Eglinton, Raphael, Smith, Hall and Pickles, 1963), the slow reacting substance of guinea-pig lung (Babilli and Vogt,

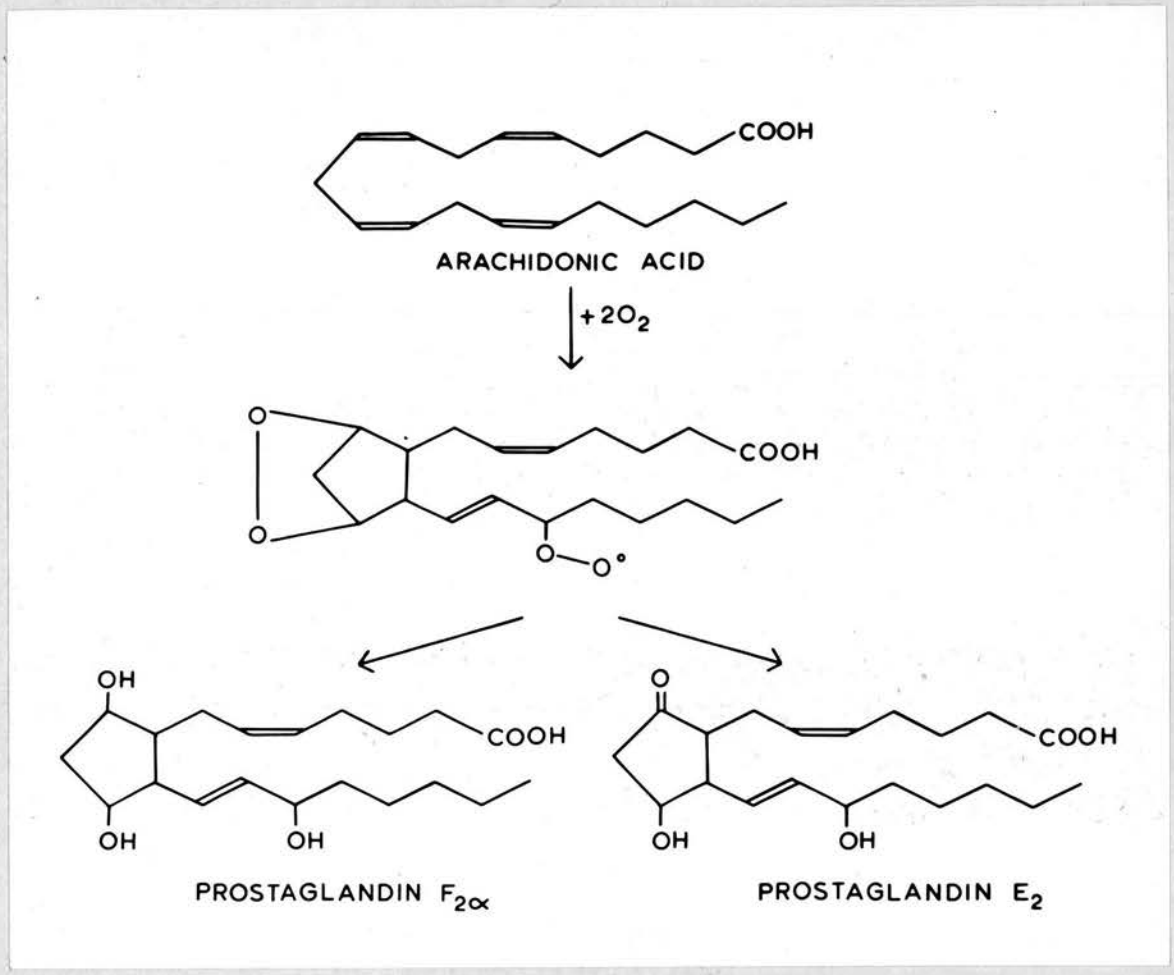

Fig. 1

The biosynthesis of prostaglandins $F_{2\alpha}$ and E_2 from arachidonic acid.

1964) and medullin (Lee, Crowshaw, Takman and Attrep, 1967). However the precise physiological roles of these "substances" is still uncertain.

An interesting observation was that prostaglandin $F_{2\alpha}$ is present in the human endometrium (Pickles, Hall, Best and Smith, 1965). However hysterectomy in the human is without effect on the time interval between consecutive ovulations, therefore the existence of a uterine luteolytic humoral mechanism in humans is extremely doubtful. The existence of a uterine luteolytic hormone in certain other mammalian species is now, however, surely beyond question. Consequently the release of prostaglandins from the uterus of the guinea-pig and sheep has been studied in order to provide evidence for or against the hypothesis that the uterine luteolytic hormone (luteolysin) is prostaglandin $F_{2\alpha}$.

SECTION 1

The Identification of Prostanglandin $F_{2\alpha}$ Released by Distension of the Guinea-pig Uterus in Vitro.

Distension of the guinea-pig uterus, in vivo, on days 2-4 of the oestrous cycle, by the insertion of two glass beads into each uterine horn, causes premature regression of the corpora lutea, with shortened oestrous cycles (Donovan and Traczyk, 1962). This effect has been attributed to the premature release of a uterine luteolytic substance (Bland and Donovan, 1966). Since prostaglandin $F_{2\alpha}$ has a luteolytic action in guinea-pigs (Blatchley and Donovan, 1969) experiments were performed to investigate whether distension of the guinea-pig uterus, in vitro, could release this prostaglandin and provide a possible explanation for the effects of bead insertion, in vivo.

Experiment 1

Methods

Incubation of Guinea-pig uterus: A female, virgin guinea-pig weighing 500 g was smeared daily by a lavage technique. Day 1 of the cycle was taken as the day before the post-ovulatory influx of leucocytes when cornification of the vagina was at a maximum. On day 3 of the cycle the guinea-pig was killed by stunning and incising the neck, the uterine horns removed and separated. One horn was distended by the intraluminal insertion of a piece of polyethylene tubing, 3 mm wide and 30 mm long. The other horn was similarly treated, except that the polyethylene tubing was removed immediately following insertion. Both horns were incubated separately at 37°C in 7 ml Tyrode's solution for three hours and gassed with 5% carbon dioxide in oxygen. Following this period, the two horns were removed and both samples of incubation fluid were stored at -20°C prior to being assayed biologically.

Assay Preparation: The rat fundal strip, prepared as described by Vane (1957), was suspended in a 5 ml organ bath in Tyrode's solution at 37°C and gassed with oxygen. Contractions of the tissue were recorded isometrically by a Grass force-displacement transducer FT03C connected through a balancing circuit to a Servoscribe pen recorder. 0.2 ml aliquot portions of the incubation fluid were added to the organ bath and assayed against a standard solution of prostaglandin E_1. A dose cycle of 4 minutes was employed with a drug contact time of 60 seconds.

Control responses to 5-hydroxytryptamine, acetylcholine and histamine were obtained. Suitable doses of the appropriate antagonists to block these responses were found. The antagonist was added to the organ bath 30 seconds before the agonist. These doses of antagonists were then added to the organ bath 30 seconds before 0.2 ml doses of the incubation fluid.

Drugs used in the Assay: (the concentration refers to the final concentration of the drug in the organ bath).

a. Standard Solution: i) Prostaglandin E_1 - Suitable doses in the range of 0.2 ng/ml to 4 ng/ml, from a standard solution containing 100 ng/ml.

b. Agonists: ii) 5-Hydroxytryptamine creatinine sulphate - 1 ng/ml

iii) Acetylcholine chloride - 2 ng/ml

iv) Histamine acid phosphate - 1 μg/ml

c. Antagonists: v) Bromo-lysergic acid diethylamide (BOL) - 20 and 100 ng/ml

vi) Atropine sulphate-50 ng/ml

vii) Mepyramine maleate-5 μg/ml

Incubation with Chymotrypsin: A 0.4 ml aliquot of the incubation fluid from the distended horn was incubated with 100 μg of chymotrypsin at 37°C for thirty minutes.

Following this period, 0.2 ml of this solution was tested on the rat fundal strip for smooth muscle contracting activity. A control dose of 100 μg chymotrypsin was tested also for such activity.

Results

The 0.2 ml dose of incubation fluid from the distended horn caused the rat fundal strip to contract, whereas that from the non-distended produced no response. The amounts of smooth muscle contracting material present were estimated to be:-

From Distended Horn $\equiv$ 65 ng PGE_1/ml

From Non-Distended Horn $\ll$ 40 ng PGE_1/ml

The contractile responses of the rat fundal strip to the 0.2 ml doses of incubation fluid from the distended horns were neither abolished nor reduced by the addition of the 2-bromolysergic acid diethylamide, atropine sulphate or mepyramine maleate. In addition, the aliquot of incubation fluid from the distended horn after incubation with chymotrypsin still continued to contract the rat fundal strip. Chymotrypsin itself did not possess any smooth muscle contracting activity in the dose used.

Conclusion

Distension of the guinea-pig uterus, *in vitro*, leads to the presence of smooth muscle contracting activity in the incubation fluid, following a three hour incubation period. This activity is not due to 5-hydroxytryptamine, acetylcholine, histamine or any of the smooth muscle contracting peptides.

Experiment 2.

Methods

Nine female guinea-pigs weighing about 500 g were killed on day 3 or 4 of the oestrous cycle, the uterine horns removed and separated. One horn from each guinea-pig was distended (test) as in experiment 1 (methods), the other horn from each being

untouched (control). Each horn was incubated in 8 ml of Tyrode's solution under the same conditions as in experiment 1. Following incubation and storage at -20°C, the incubation fluid from each horn, test and control, was assayed on the rat fundal strip for smooth muscle contracting activity.

Solvent Extraction Procedure: The individual test samples and control samples were then pooled separately to give one "test" and one "control" sample. These were treated in parallel by a solvent extraction method for the isolation of prostaglandin-like material (Bergström and Samuelsson, 1963; Horton and Main, 1967) (See Fig. 2). Each sample was taken to pH 3 with hydrochloric acid and partitioned twice with an equal volume of ethyl acetate. The two ethyl acetate fractions were combined, reduced in volume to 20 ml by reduced pressure evaporation and partitioned twice with 20 ml 0.1 M pH8 phosphate buffer. The 40 ml of buffer was adjusted to pH3 with hydrochloric acid and partitioned twice with 40 ml ethyl acetate. The ethyl acetate fraction was evaporated to dryness, under reduced pressure, the residue dissolved in 15 ml 67% ethanol and washed twice with 15 ml petroleum spirit (bp. 60-80°C). The ethanol fraction was then evaporated to dryness, under reduced pressure, the residue dissolved in 2 ml water. The two final extracts (test and control) were then assayed on the rat fundal strip.

Additional Control Experiments: A uterine horn, removed from a guinea-pig on day 3 of the oestrous cycle, was incubated in the presence of a piece of polyethylene tubing, though not distended by it. The other uterine horn from the guinea-pig was incubated alone. (Conditions of incubation as in experiment 1 - methods). Following the three hour period, the incubation fluid from both horns was tested on the rat fundal strip for smooth muscle contracting activity.

Also, two pieces of polyethylene tubing were incubated alone, in the absence of uterine tissue. Again the incubation fluid was tested on the rat fundal strip for smooth muscle contracting activity.

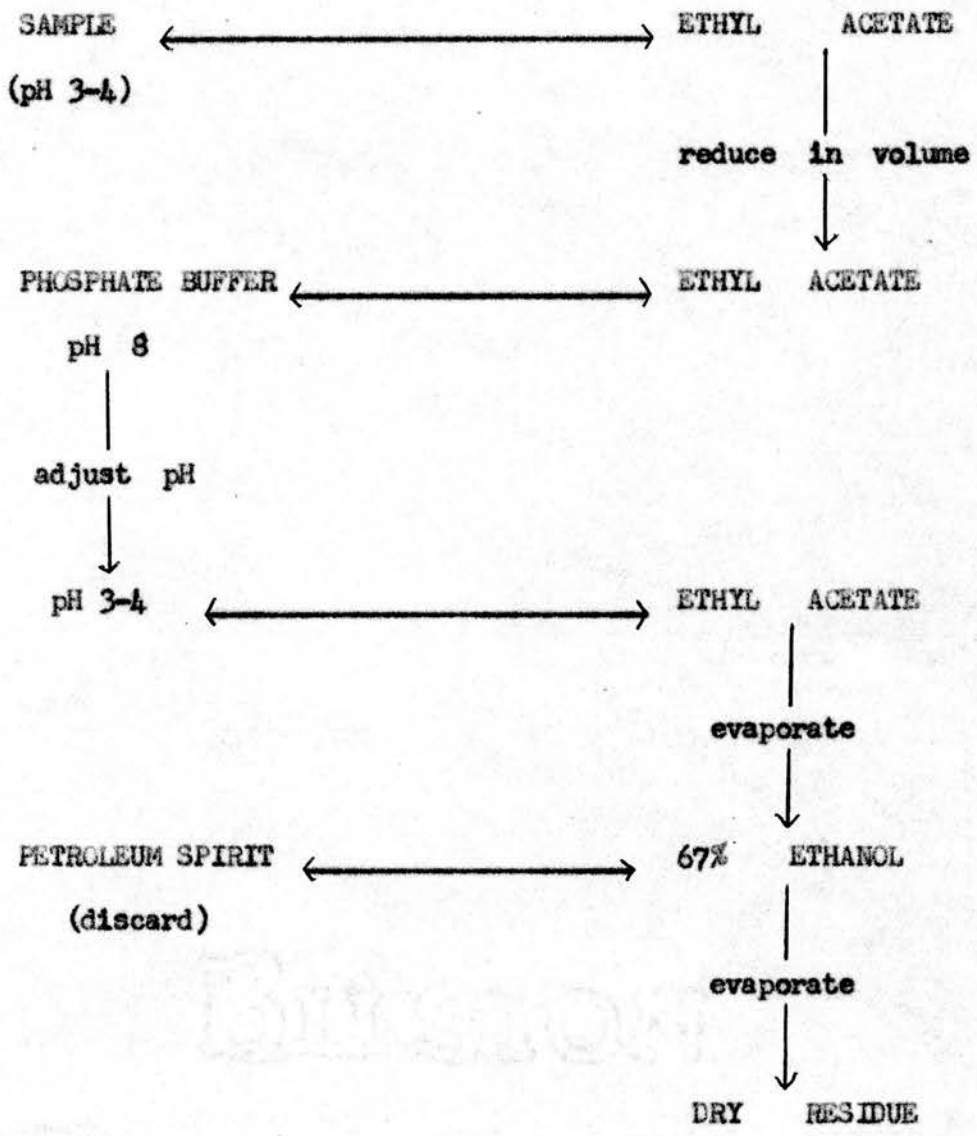

Fig. 2

Solvent extraction procedure for the isolation of prostaglandin-like material (i.e. polar acidic lipids).

Assay Preparation and Drugs Used: The rat fundal strip was employed under the same conditions as in experiment 1, (methods). All samples were assayed against a standard solution of prostaglandin E_1 containing 100 ng/ml.

Results

In eight out of nine instances, the amount of smooth muscle contracting activity in the incubation fluid from the distended horn exceeded that from the non-distended horn (see Table 1.)

SAMPLE	ACTIVITY ≡ ng PGE_1/ml	
	Test (Distended horn)	Control (Non-distended horn)
1	12	7.5
2	10	2.5
3	15	n.d (<3)
4	10	10
5	14	n.d (<3)
6	16	n.d (<3)
7	18	7
8	14	7
9	20	n.d (<3)

n.d = no detectable activity.

Table 1: Results of the Assay of the Nine Pairs of Incubated Samples Obtained in Experiment 2, on the Rat Fundal Strip

After the pooling of samples, followed by subsequent solvent extraction and assaying on the rat fundal strip, the test sample (from the distended horns) contained smooth muscle activity equivalent to 40 ng prostaglandin E_1 whereas the control sample (from the non-distended horns) contained no detectable activity (< 10 ng prostaglandin E_1).

In the additional control experiments there was no difference in prostaglandin concentration ($\equiv$5 ng prostaglandin E_1 /ml) between the incubation medium obtained from the uterine horn incubated alone and that incubated in the presence of polyethylene tubing. Also no smooth muscle contracting activity could be detected in the incubation media from the two pieces of polyethylene tubing.

Conclusion

Distension of the guinea-pig uterus by a piece of polyethylene tubing, followed by a period of incubation, *in vitro*, leads to the presence of smooth muscle contracting activity in the incubation medium, the active principle of which behaves like a prostaglandin in its solvent partitioning and pharmacological properties. The presence of such activity cannot be attributed to an interaction between the polyethylene tubing and uterine tissue, nor due to the leaching of the polyethylene tubing itself.

Experiment 3

Methods

Nine female guinea-pigs weighing about 500 g were killed on day 3 of the oestrous cycle, the uterine horns were dissected out and incubated as in experiment 2, (methods). As an additional precaution any intraluminal contents of the distended horns remaining were washed away before incubation so that any activity detected was not due to substances present in the lumen. Following incubation and storage at -20°C, the incubation fluid from each horn, distended and non-distended, was assayed on the rat fundal strip, against a standard solution of prostaglandin E_1 (100 ng/ml) for smooth muscle contracting activity. After assaying, the individual samples from the distended and non-distended horns were pooled separately to give one "test" and one "control" sample. These two samples were then treated by the solvent extraction procedure for the isolation of prostaglandin-like material (experiment 2, methods). The final dry residue

was dissolved in 0.5 ml 30% ethyl acetate in benzene and subjected to silicic acid column chromatography for the separation of the various prostaglandins.

Silicic Acid Column Chromatography: This method for separating the different series of prostaglandins was first described by Samuelsson (1963). 3 g of silicic acid (Bio-Rad Labs. minus 325 mesh) was activated by heating at 110°C for one hour. After being allowed to cool in a desiccator, the silicic acid was suspended in a heavy petroleum spirit (bp. 60-80°C) and the slurry poured into a glass column of internal diameter 1 cm. This gave a column of silicic acid approximately 9 cm in length. The column was then washed with 30% ethyl acetate in benzene.

The test and control samples were applied to such columns in a dropwise manner with a Pasteur pipette. The flasks were rinsed with portions of the first 20 ml of 30% ethyl acetate in benzene used to elute the columns. The columns were eluted under negative pressure, in a stepwise manner with increasing concentrations of ethyl acetate in benzene. Concentrations of ethyl acetate used were 30%, 40%, 80% and 100%, followed by a final elution of the column with methanol. The flow rate was approximately 1 ml per minute. Column eluate fractions were evaporated to dryness, under reduced pressure, and the residues dissolved in 1 ml distilled water for assaying on the rat fundal strip for smooth muscle contracting activity. Fractions 1 - 7 were assayed against a standard solution of prostaglandin E_1 (100 ng/ml) whilst fractions 8 - 10 were assayed against a similar solution of prostaglandin $F_{2\alpha}$.

Results

Table 2 shows the amount of smooth muscle contracting activity present in the incubation fluid from the distended and non-distended horns following incubation and prior to pooling. In all nine instances the activity released from the distended horn exceeds that from the non-distended horn.

SAMPLE	ACTIVITY ≡ ng PGE_1/ml	
	Test (Distended horn)	Control (Non-distended horn)
1	25	n.d (< 5)
2	20	n.d (< 5)
3	7.5	2
4	7.5	5
5	13	3
6	9	n.d (< 5)
7	7	n.d (< 5)
8	33	3
9	9	4

n.d = no detectable activity.

Table 2: Results of the Assay of the Nine pairs of Incubated Samples Obtained in Experiment 3, on the Rat Fundal Strip

The results of the silicic acid column chromatography of the extracted, pooled samples are shown in Table 3.

Fraction Number	Eluant % Et. Ac. in Benzene	Activity on Rat Fundal Strip	
		Test	Control
1	20ml 30%	n.d	n.d
2	40ml 30%	n.d	n.d
3	20ml 40%	n.d	n.d
4	20ml 40%	n.d	n.d
5	170ml 40%	25 ng PGE_1	25 ng PGE_1
6	20ml 40%	n.d	n.d
7	20ml 40%	n.d	n.d
8	100ml 80%	40 ng PGF_2	15 ng PGF_2
9	40ml 100%	50 ng PGF_2	7.5 ng PGF_2
10	50ml Methanol	≡15 ng PGF_2	≡15 ng PGF_2

n.d = no detectable activity

Table 3: Results of the Assay on the Rat Fundal Strip of the Fractions Obtained from the Silicic Acid Columns in Experiment 3

Holmes and Horton (1968) showed that with this particular batch of silicic acid, prostaglandins of the "E" series were eluted by 40% ethyl acetate in benzene whilst those of the "F" series by 80%. Table 3 shows that prostaglandin E-like and prostaglandin F-like material is released from both the distended and non-distended horns. However, whilst the amounts of prostaglandin E-like material are identical, there is more than twice as much prostaglandin F-like material produced by the distended horns. The amounts produced, though, are small.

Conclusion

Incubation of the distended guinea-pig uterus, in vitro, leads to the presence of smooth muscle contracting activity in the incubation medium. After organic solvent extraction, the polar acidic lipids isolated can be resolved into prostaglandin E-like and prostaglandin F-like material. The incubation of non-distended uterine horns leads in some instances to smooth muscle contracting activity in the incubation medium also, though in smaller quantities. This activity can be resolved similarly into prostaglandin - like material of the "E" and "F" series. However, whereas the amounts of prostaglandin E-like material released from the distended and non-distended horns are identical, the distended horns produced more than twice as much prostaglandin F-like material. The amounts released were too small to permit further identification.

Experiment 4

This experiment was designed to assess quantitatively the release of the "active material" from the distended and non-distended uterine horns with time.

Methods

Four guinea-pigs were killed on day 3 of the oestrous cycle, the uterine horns dissected out and separated. One horn from each animal was distended as in experiment 1 (methods) and all eight horns were incubated separately in 10 ml of Tyrode's solution,

aerated with oxygen, at 37°C. 0.2 ml aliquot portions were taken every two hours and assayed on the rat fundal strip for smooth muscle contracting activity against a standard solution of prostaglandin $F_{2\alpha}$ (100 ng/ml).

Results

The assay results for the amount of "activity" released by the four individual distended horns have been pooled, as have those for the non-distended horns and are represented in Figure 3.

The initial release of the "active material" from the distended horns is small, though there is a large release between two and four hours of incubation. Smaller increases occur between four and six hours respectively. No "active material" is released from the non-distended horns until after four hours of incubation. However, there is quite a large rise after six hours.

Conclusion

There is an increase in the total amount of active material released with time. The total amount released from the distended horns at any one time is greater than the amount released from the non-distended horns. The actual mechanism by which polyethylene tube insertion promotes the release of "active material" from the uterus was not studied and is, so far, unknown. It may be noted that the mechanism by which uterine bead insertion, in vivo, causes the premature release of a luteolytic substance from the uterus is also unknown. In this experiment as in the other experiments in this series, care was taken not to damage the uterus whilst inserting the tubing thus minimising tissue damage as a contributory cause to the effect produced. However, the large rise in "active material" released from the non-distended horns after six hours may be due in part, to tissue breakdown following prolonged incubation.

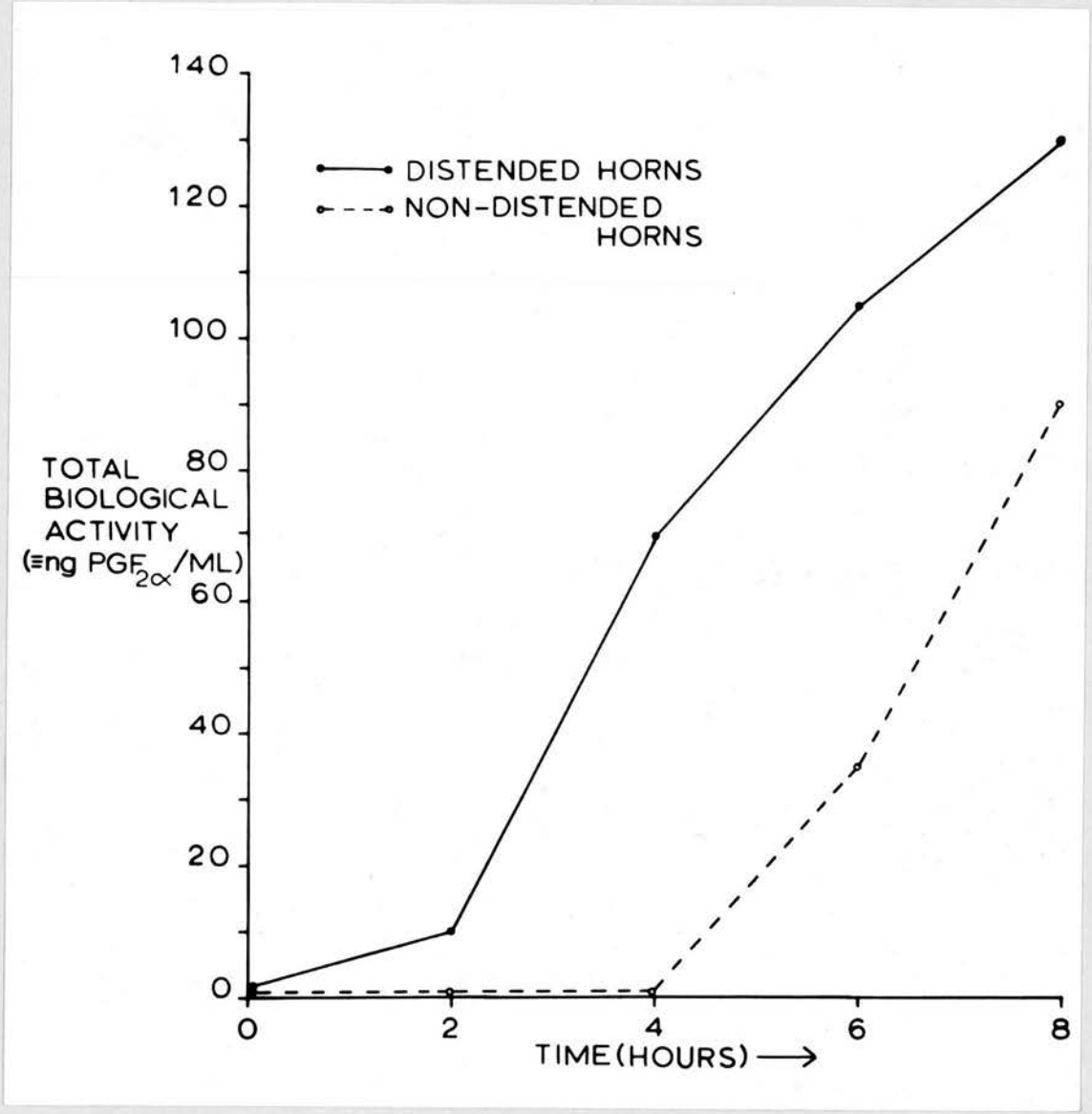

Fig. 3

Total biological activity released by distended and non-distended uterine horns during an eight hour incubation. Aliquot portions were taken every two hours and assayed on the rat fundal strip (experiment 4).

Experiment 5

In this experiment an attempt was made to identify more conclusively the "active material" released by distension of the uterus.

Methods

Thirty-five guinea-pigs were killed on day 3 of the oestrous cycle, the uterine horns dissected out and separated. One horn from each animal was distended as in experiment 1 (methods), the other horn was left untouched and acted as the control. Each horn was incubated in 10 ml Tyrode's solution, gassed with oxygen at 37°C for 5 hours. The fluid obtained from the incubated samples was pooled so as to give one "test" sample (from the distended horns) and one "control" sample (from the non-distended horns). Both samples were extracted by the solvent extraction procedure as in experiment 2 (methods), followed by silicic acid column chromatography.

Silicic Acid Column Chromatography: Columns were prepared and run in a similar manner as in experiment 3 (methods). However a different batch of silicic acid was used (Sigma SIL-R, low grade 100 mesh). Experience has shown that variations in "retention" properties occur among different batches of silicic acid. Consequently a control column using authentic prostaglandins was first prepared and run as follows.

4.5 g of the silicic acid was activated by heating in an oven at 110°C for one hour. After being allowed to cool, the silicic acid was suspended in heavy petroleum spirit (b.p. 60-80°C) and poured into a glass column of 1 cm diameter to give a column of silicic acid about 9 cm in length. The column was then washed with 30% ethyl acetate in benzene. 2.5 μg each of prostaglandin E_1 and prostaglandin $F_{2\alpha}$ dissolved in 0.5 ml 30% ethyl acetate in benzene, were then applied to the column in a dropwise manner using a Pasteur pipette. The flask was rinsed with aliquot portions of the first fraction of eluant. The column was eluted under negative pressure with increasing concentrations of ethyl acetate in benzene as shown in Table 4. Each fraction

was 40 ml in volume. Elution of the column was completed by a 50 ml fraction of methanol (fraction 17).

Fraction Number	% of Ethyl Acetate in Benzene
1	30
2	40
3	40
4	50
5	50
6	60
7	60
8	60
9	60
10	70
11	70
12	80
13	80
14	80
15	80
16	100

Table 4: Percentages of Ethyl Acetate in Benzene used to Elute Authentic Prostaglandins from a Silicic Acid column in Experiment 5

The fractions obtained from the column were evaporated to dryness, under reduced pressure and assayed on the rat fundal strip. Fractions 1 - 10 were assayed against a standard solution of prostaglandin E_1 (100 ng/ml) whilst fractions 11 - 17 were assayed against a similar solution of prostaglandin $F_{2\alpha}$.

After the results of running this column were obtained, the solvent extracted "test" and "control" samples were applied to similar columns and eluted under negative pressure with the volumes and concentrations of ethyl acetate in benzene as shown in Table 5.

Fraction Number	Volume of Eluant (ml)	% Ethyl Acetate in in Benzene
1	40	30
2	40	40
3	20	60
4	110	60
5	20	60
6	150	80
7	40	100
8	50	Methanol

Table 5: Volumes of Eluant and Percentages of Ethyl Acetate in Benzene used in the Silicic Acid Column Chromatography of the "Test" and "Control" Samples in Experiment 5

The fractions obtained from the columns were evaporated to dryness, under reduced pressure, the residue dissolved in 1 ml of water and assayed on the rat fundal strip for smooth muscle contracting activity. Fractions 1 - 5 were assayed against a standard solution of prostaglandin E_1 (100 ng/ml), whilst fractions 6 - 8 were assayed against a similar solution of prostaglandin $F_{2\alpha}$.

Parallel Bio-Assay: Following the assay on the rat fundal strip, fraction 6 from both columns was assayed on three further biological preparations against standard solutions of prostaglandin E_1 and prostaglandin $F_{2\alpha}$ (100 ng/per ml or 1 μg per/ml depending upon the sensitivities of the tissues to each prostaglandin). The assay preparations used were:-

i) Rabbit jejunum - A piece of rabbit jejunum about one inch long was suspended in a 5 ml organ bath in Tyrode's solution, bubbled with air, at 37°C. Contractions of the tissue were recorded isometrically using a Grass force displacement transducer FT03C, connected through a balancing circuit to a Servoscribe pen recorder. A dose

cycle of 4 minutes was employed with a drug contact time of 60 seconds.

ii) Jird colon - A piece of ascending colon, about 1½" long, from the Mongolian jird (Meriones unguiculatus) was suspended in a 2 ml organ bath in the de Jalon's colon Ringer solution, bubbled with oxygen, at 30°C. Contractions of the tissue were recorded isotonically by means of a frontal writing lever on a smoked drum. A dose cycle of four minutes was used with a drug contact time of 60 seconds.

iii) Guinea-pig ileum - A piece of guinea-pig ileum, about one inch in length, was suspended in a 5 ml organ bath in Tyrode's solution, bubbled with air, at 37°C. Contractions of the tissue were recorded isotonically by means of a frontal writing lever on a smoked drum. A dose cycle of four minutes was used with a drug contact time of 60 seconds.

Gas Chromatography/Mass Spectrometry: Following the parallel bioassay, fraction 6 from the "test" column only was subjected to analysis by combined gas chromatography/mass spectrometry (Thompson, Los and Horton, 1970). For this procedure, derivative formation is necessary.

This fraction was evaporated to dryness in a desiccator, in vacuo, the residue dissolved in 0.2 ml of methanol and transferred into a 0.5 ml stoppered tube. The flask was rinsed with a further 0.2 ml of methanol and the washings added to the stoppered tube. The methanol was then evaporated off by a fine jet of air. The residue remaining was methylated by a reaction for 30 minutes with a freshly prepared solution of diazomethane in diethyl ether-methanol (9:1). Following this reaction period, the excess solution was vaporised again by the use of a fine jet of air. The trimethylsilyl ether was then formed by the addition of 25 μl bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) to the methyl ester, a reaction taking three hours to go to completion. The methyl ester/trimethylsilyl ether (Me/TMS) of authentic prostaglandin $F_{2\alpha}$ (500 ng) was prepared also in a similar manner. 10 µl quantities of each sample were injected

on to the gas chromatographic column without removal of the BSTFA.

Analysis was performed on an LKB 9000 gas chromatograph-mass spectrometer. The column (1.5 m x 1.5 mm i.d.) was packed with 3% OV 1 on Supasorb AW, 100-120 mesh, pre-treated with dimethylchlorosilane in carbon tetrachloride. The column temperature was 200°C. The carrier gas was helium which flowed at 20 ml per minute. The mass spectra were recorded at an electron voltage of 27.5.

A 10 μl sample of the Me/TMS derivative of authentic prostaglandin $F_{2\alpha}$ was injected on to the column. Its retention time, taken from the gas chromatographic recording, was noted. A mass spectrum was taken at this time. The same derivative of fraction 6 from the "test" sample was then injected on to the column. A mass spectrum of the effluent from the column was taken at the previously noted retention time for the Me/TMS derivative of authentic prostaglandin $F_{2\alpha}$.

Further Purification of "Test" Sample: The remnants (15 μl) of the "reacted" fraction 6 from the "test" column were evaporated to dryness in a desiccator in vacuo, and re-extracted. The residue was dissolved in 20 ml of 67% ethanol and washed twice with 20 ml portions of petroleum spirit (b.p. 60°C - 80°C). The ethanol fraction was evaporated to dryness under reduced pressure, the residue dissolved in 0.5 ml 30% ethyl acetate in benzene and subjected to silicic acid column chromatography, as described previously in this experiment. The fractions obtained from the columns were evaporated to dryness under reduced pressure, the residues dissolved in 1 ml of water and assayed on the rat fundal strip for smooth muscle contracting activity. Fractions 1 - 5 were assayed against a standard solution of prostaglandin E_1 (100 ng/ml), while fractions 6 - 8 were assayed against a similar solution of prostaglandin $F_{2\alpha}$. Fraction 6 from the column was then subjected to analysis be combined gas chromatography and mass spectrometry as described previously.

Results

i) Result of the Silicic Acid Column Chromatography Using Authentic Prostaglandins

The result is shown in Figure 4. Prostaglandin E_1 was totally eluted from the column by 60% ethyl acetate in benzene (fractions 6 - 10). The second fraction of 70% ethyl acetate in benzene began to elute the prostaglandin $F_{2\alpha}$ (fraction 11) but the majority was eluted by the two initial fractions of 80% ethyl acetate in benzene (fractions 12 and 13). Elution of the column was completed by fraction 15.

ii) Results of the Silicic Acid Column Chromatography of the "Test" and "Control" Samples

The results are shown in Figure 5. Based on the results of the column using authentic prostaglandins, fraction 4 (110 ml of 60% ethyl acetate in benzene) elutes prostaglandins of the "E" series from the column, whilst fraction 6 (150 ml of 80% ethyl acetate in benzene) elutes prostaglandins of the "F" series. As can be seen, the amount of prostaglandin E-like material in the "test" and "control" samples is small and nearly identical. There is activity equivalent to 30 ng prostaglandin E_1 in the "test" sample and activity equivalent to 25 ng prostaglandin E_1 in the "control". However, there is a large difference in prostaglandin F-like activity. There is activity equivalent to 1 μg of prostaglandin $F_{2\alpha}$ in the test sample but activity equivalent to only 100 ng in the control, a ten-fold difference. There was a small amount of activity also in the methanol fraction from both columns.

iii) Results of the Parallel Bio-Assay

Table 6 shows the results of the bio-assays of fraction 6 from the "test" and "control" columns on the rat fundal strip, rabbit jejunum, ascending colon of the mongolian jird and the guinea-pig ileum.

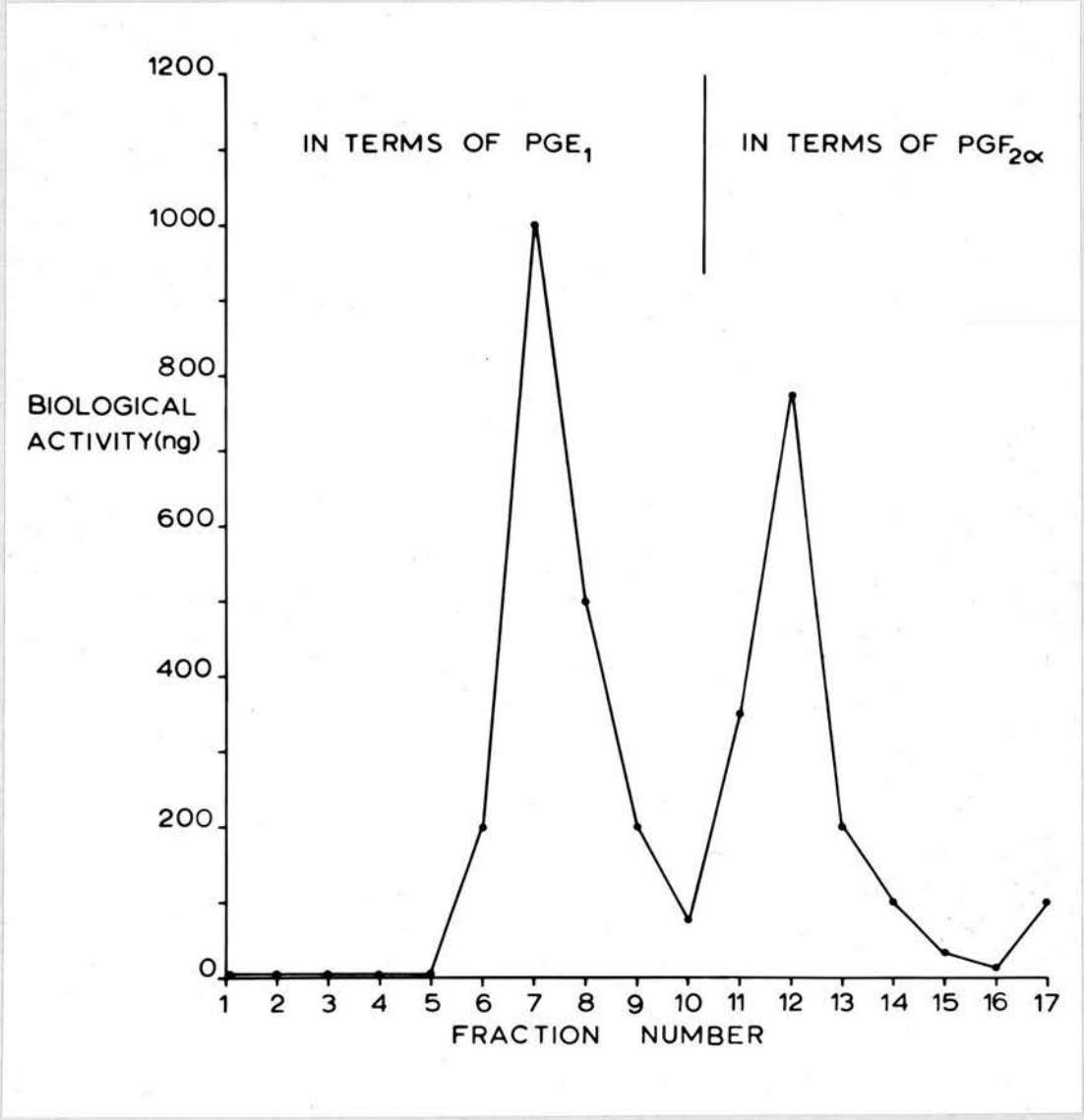

Fig. 4

Results of the bioassay on the rat fundal strip of the silicic acid column chromatography using authentic prostaglandins (experiment 5).

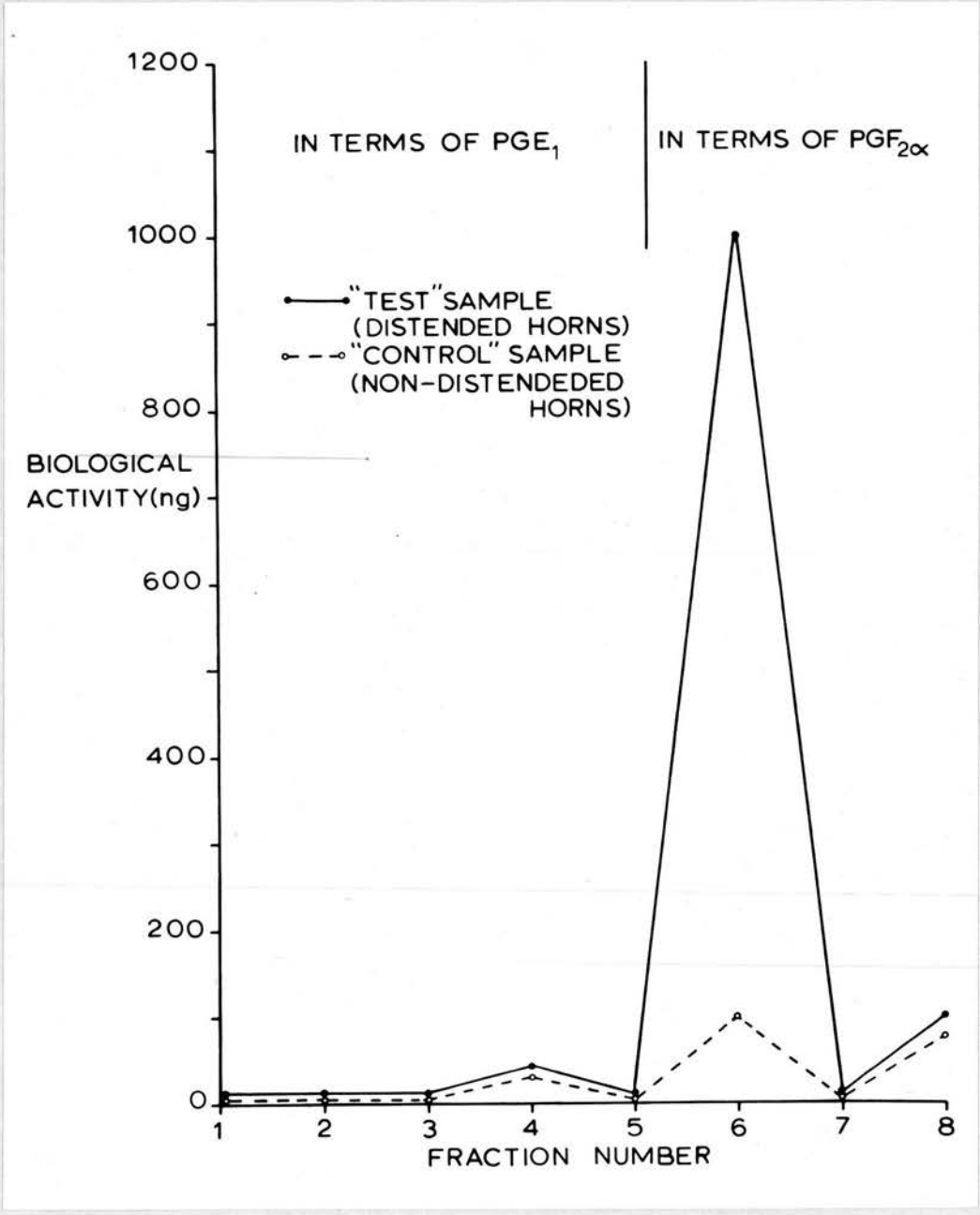

Fig. 5

Results of the bioassay on the rat fundal strip of the silicic acid column chromatography of the "test" sample (from distended uterine horns) and "control" sample (from non-distended horns) (experiment 5).

Assay Preparation	Activity in "Test" Sample (μg)		Activity in Control Sample (μg)	
	$PGF_{2\alpha}$	PGE_1	$PGF_{2\alpha}$	PGE_1
Rat Fundus	1.0	1.5	0.10	0.15
Rabbit Jejunum	1.0-2.0	10-20	0.063-0.125	0.63-1.25
Jird Colon	1.2-1.4	0.60-0.70	0.087-0.100	0.044-0.500
Guinea-pig Ileum	1.3-2.0	0.067-0.100	Insufficient to assay	

Table 6: Results of the Parallel Bio-Assay of Fraction 6 Obtained from the Silicic Acid Columns in Experiment 5, Assayed against Prostaglandin E_1 (PGE_1) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on Four Preparations.

The estimated amount of activity in fraction 6 from the "test" column was similar on all four tissues when assayed in terms of prostaglandin $F_{2\alpha}$ (greatest index of discrimination = 2). However, the estimates differed widely when the sample was assayed in terms of prostaglandin E_1 (greatest index of discrimination = 297). In addition, qualitative differences in contractions of the rabbit jejunum and guinea-pig ileum elicited by prostaglandins E_1 and $F_{2\alpha}$ were observed. The response to E_1 tended to be quick in onset but shorter in duration. The response to prostaglandin $F_{2\alpha}$ on the other hand was slower in onset but of longer duration. The responses produced by fraction 6 from the "test" column resembled those produced by prostaglandin $F_{2\alpha}$.

Fraction 6 from the "control" column produced a similar set of results. The amount of activity present, however, was 10-12 times less than that in fraction 6 from the "test" column.

iv) Results of the Gas Chromatography/Mass Spectrometry

The methyl ester/trimethyl ether (Me/TMS) of authentic prostaglandin $F_{2\alpha}$ on gas chromatography had a retention time of 16.0 minutes. A mass spectrum was taken at this time. The Me/TMS derivative of fraction 6 from the "test" column contained many substances as was shown by the gas chromatographic recording. The trace of the mass spectrum taken of the effluent from the column at 16.0 minutes had many $^{m}/e$ peaks. However, those peaks characteristic of the Me/TMS derivative of authentic prostaglandin $F_{2\alpha}$ at $^{m}/e$ values of 494, 513, 569 and 584 were absent.

v) Results following further purification of the "test" sample

After purifying the "test" sample further the results of the silicic acid column chromatography indicated that there was still prostaglandin F-like activity, equivalent to 100 ng prostaglandin $F_{2\alpha}$ (See Table 7).

Fraction Number	Eluant % Et Ac in Benzene	Activity on Rat Fundal Strip
1	40 ml 30%	< 25 ng PGE_1
2	40 ml 40%	< 25 ng PGE_1
3	20 ml 60%	< 25 ng PGE_1
4	110 ml 60%	< 25 ng PGE_1
5	20 ml 60%	< 25 ng PGE_1
6	150 ml 80%	100 ng $PGF_{2\alpha}$
7	40 ml 100%	< 50 ng $PGF_{2\alpha}$
8	50 ml Methanol	50 ng $PGF_{2\alpha}$

Table 7: Results of the Assay on the Rat Fundal Strip of the Fractions Obtained from the Silicic Acid Column following Further Purification of the "Test" Sample in Experiment 5.

The Me/TMS derivative of authentic prostaglandin $F_{2\alpha}$ on gas chromatography had a retention time of 16.1 minutes. The trace of the mass spectrum taken at this time

contained the $^{m}/e$ peaks typical of prostaglandin $F_{2}\alpha$. Figure 6 shows the ten peaks of $^{m}/e$ above 300 on which positive identification was made. These peaks occur at $^{m}/e$ values of 307, 333, 353, 379, 404, 423, 494, 513, 569 and 584. Peaks below $^{m}/e$ 300 tend to be lost in the background "noise" and are therefore of little value in affording identification. The Me/TMS derivative of the material in fraction 6 from the silicic acid column on gas chromatographic analysis produced a peak at 16.1 minutes. The trace of the mass spectrum taken on the effluent from the column at this time contained the ten peaks of $^{m}/e$ above 300 characteristic of prostaglandin $F_{2}\alpha$. This evidence indicates that prostaglandin $F_{2}\alpha$ is present in the "test" sample. Other prominent peaks occurring in the mass spectrum at $^{m}/e$ 323, 339, 361, 380, 437 and 451 are due to the presence of interfering substances.

Conclusion

Distension of the guinea-pig uterus by the intraluminal insertion of a piece of polyethylene tubing leads to the release of prostaglandin F-like material. The amount released is ten times greater than that released from non-distended horns. The amount of prostaglandin E-like material released from the distended uterine horns is much smaller and is nearly identical to that released from non-distended horns. The parallel bio-assays confirmed that the material present in fraction 6 from each silicic acid column closely resembled prostaglandin $F_{2}\alpha$ but could not be prostaglandin E_{1}, as reflected in the indices of discrimination of 2 and 297 respectively.

At the first attempt, combined gas chromatography and mass spectrometry failed to confirm that the material in fraction 6 from the 'test' column was prostaglandin $F_{2}\alpha$. This was due to the presence of large amounts of contaminating substances which probably interfered with the chemical reactions involved in derivative formation. After further purification, however, analysis by gas chromatography and mass spec-

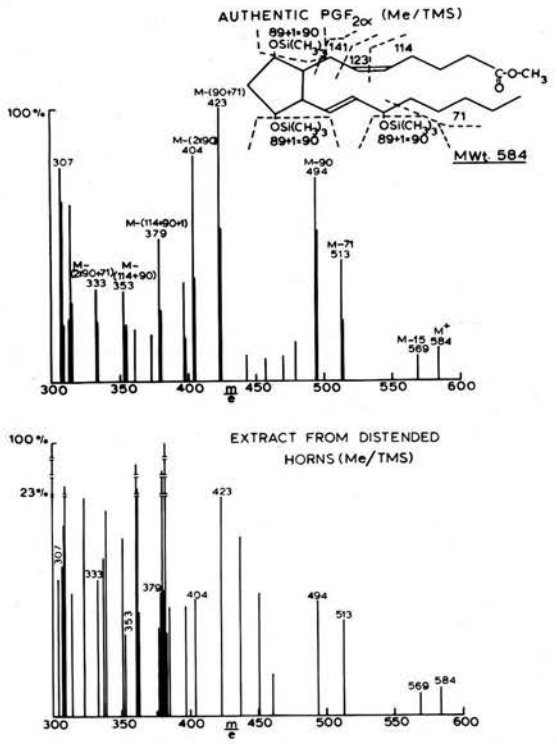

Fig. 6

Line diagrams of mass spectra ($^{m}/e$ peaks greater than 300) of the Me/TMS derivative of material released from distended uterine horns and of authentic prostaglandin $F_{2}\alpha$ (experiment 5). Ordinate: percentage relative intensity of peaks. Abscissa: m/e value of peaks.

trometry confirmed the presence of prostaglandin $F_{2\alpha}$ in the 'test' sample (from the distended horns). The amounts of prostaglandin E-like material released were too small for further identification.

Experiment 6

In the previous experiment combined gas chromatography and mass spectrometry failed in the first instance to confirm the presence of prostaglandin $F_{2\alpha}$ in the 'test' sample and in the second instance no 'control' sample was included. This experiment was therefore repeated.

Methods

Thirty guinea-pigs on day 3 of the oestrous cycle were killed, their uterine horns removed and separated. One horn from each animal was distended by the intraluminal insertion of a piece of polyethylene tubing, the other horn was left untouched and acted as the control. All horns were incubated separately in 10 ml Tyrode's solution, aerated with oxygen, at 37°C for 5 hours. Following this period, the incubation fluid from the distended horns was pooled to give one "test" sample. Likewise the incubation fluid from the non-distended horns was pooled to give one "control" sample. Both pooled samples were then subjected to the solvent extraction procedure as in experiment 2 (methods). The final fractions from the column were evaporated to dryness under reduced pressure, the residues dissolved in 1 ml water and assayed on the rat fundal strip for smooth muscle contracting activity. Fractions 1 - 5 were assayed against a standard solution of prostaglandin E_1 (100 ng/ml) whilst fractions 6 - 8 were assayed against a similar solution of prostaglandin $F_{2\alpha}$.

Gas Chromatography/Mass Spectrometry: Fractions 6 from the "test" and "control" columns were subjected to analysis by combined gas chromatography and mass spectrometry as in experiment 5 (methods), except that two derivatives of each fraction were

prepared. The fractions were taken to dryness in a desiccator, in vacuo, the residues dissolved each in 0.4 ml methanol and divided into two equal portions. One portion of each was used to form the Me/TMS derivative as described previously (experiment 5, methods). The other half was used to form the methyl ester/trifluoroacetate (Me/TFA). The methyl ester was prepared first. The trifluoroacetate was prepared by reacting the methyl esters for 2 hours with 200 μl of trifluoroacetic anhydride which was then removed in a desiccator, in vacuo. The residues remaining were dissolved in 25 μl of hexane.

The Me/TMS and Me/TFA derivatives of authentic prostaglandin $F_{2\alpha}$ (500 ng) were prepared also. 10 μl samples of all derivatives were injected on to the gas chromatography column. The column temperature was 190°C for the Me/TFA compounds and 200°C for the Me/TMS derivatives. Other column conditions were the same as in experiment 5 (methods). Mass spectra, taken at the appropriate retention times, were recorded at an electron voltage of 27.5.

Results and Conclusion

i) Result of the Silicic Acid Column Chromatography

The result is represented in Figure 7 and is similar to that obtained in experiment 5. The amount of prostaglandin E-like activity released from the distended and non-distended horns is small and nearly identical ($\equiv$ 80 ng prostaglandin E_1), whereas there is a large difference in the amount of prostaglandin F-like activity. There is activity equivalent to 700 ng prostaglandin $F_{2\alpha}$ in the "test" sample (from the distended horns) but activity equivalent to only 80 ng prostaglandin $F_{2\alpha}$ in the "control" (from the non-distended horns), again a notable difference.

ii) Results of the Gas Chromatography/Mass Spectrometry

The Me/TMS and Me/TFA derivatives of authentic prostaglandin $F_{2\alpha}$ on gas chromatography produced peaks having retention times of 19 minutes and 6 minutes respectively. The

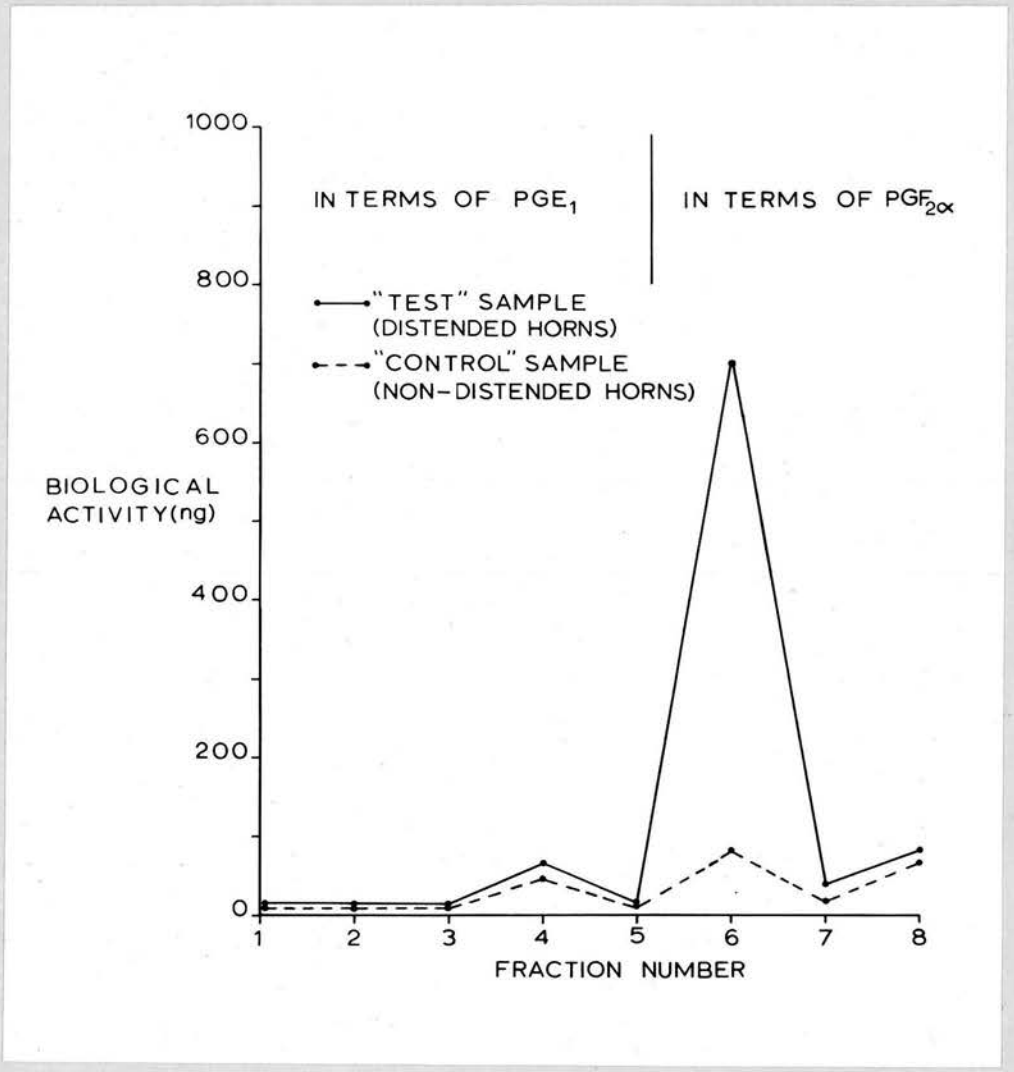

Fig. 7

Results of the bioassay on the rat fundal strip of the silicic acid column chromatography of the "test" sample (from distended uterine horns) and "control" sample (from non-distended uterine horns) (experiment 6).

same derivatives of fraction 6 from the "test" column produced similar peaks at identical retention times in amounts agreeing with the bioassay result. The same derivatives of fraction 6 from the "control" column, however, did not produce any peaks at these retention times, since the amounts present were subthreshold for detection. Mass spectra were taken at the appropriate retention times irrespective of whether there was a gas chromatographic peak or not. Line diagrams of these spectra showing peaks of $^{m}/e$ above 300 are shown in Figure 8.

On inspection of the Me/TMS derivatives first, in fraction 6 from the "control" column there are 4 prominent peaks at $^{m}/e$ 361, 382, 437 and 451. These peaks are present also in fraction 6 from the "test" column but, in addition, there are ten other peaks at $^{m}/e$ 307, 333, 353, 379, 404, 423, 494, 513, 569 and 584. These are the peaks characteristic of the Me/TMS derivative of authentic prostaglandin $F_{2}\alpha$. A similar pattern is seen in the Me/TFA compounds. There is one common peak in both fractions at $^{m}/e$ 326. Fraction 6 from the "test" column has three additional prominent peaks at $^{m}/e$ 314, 428 and 542. These are the three peaks characteristic of the Me/TFA derivative of prostaglandin $F_{2}\alpha$.

On this evidence of gas chromatography and mass spectrometry performed on two derivatives, it can be concluded that the "test" sample (from the distended horns) contains prostaglandin $F_{2}\alpha$, whilst no detectable amounts are present in the "control" sample (from the non-distended horns). The common peaks occurring in the mass spectra of both derivatives are due to the same impurities present in approximately equal amounts in each sample. In addition, on gas chromatographic evidence, no prostaglandin $F_{1}\alpha$ (retention times; Me/TMS derivative 21 minutes, Me/TFA derivative 7 minutes) nor 5α, 7α, - dihydroxy-11-oxo-tetranorprostanoic acid (the main urinary metabolite of prostaglandin $F_{2}\alpha$ in the guinea-pig; retention times, Me/TMS derivative 7 minutes, Me/TFA derivative 4.25 minutes) could be detected in either sample, since no gas chromotographic peaks occurred at these retention times.

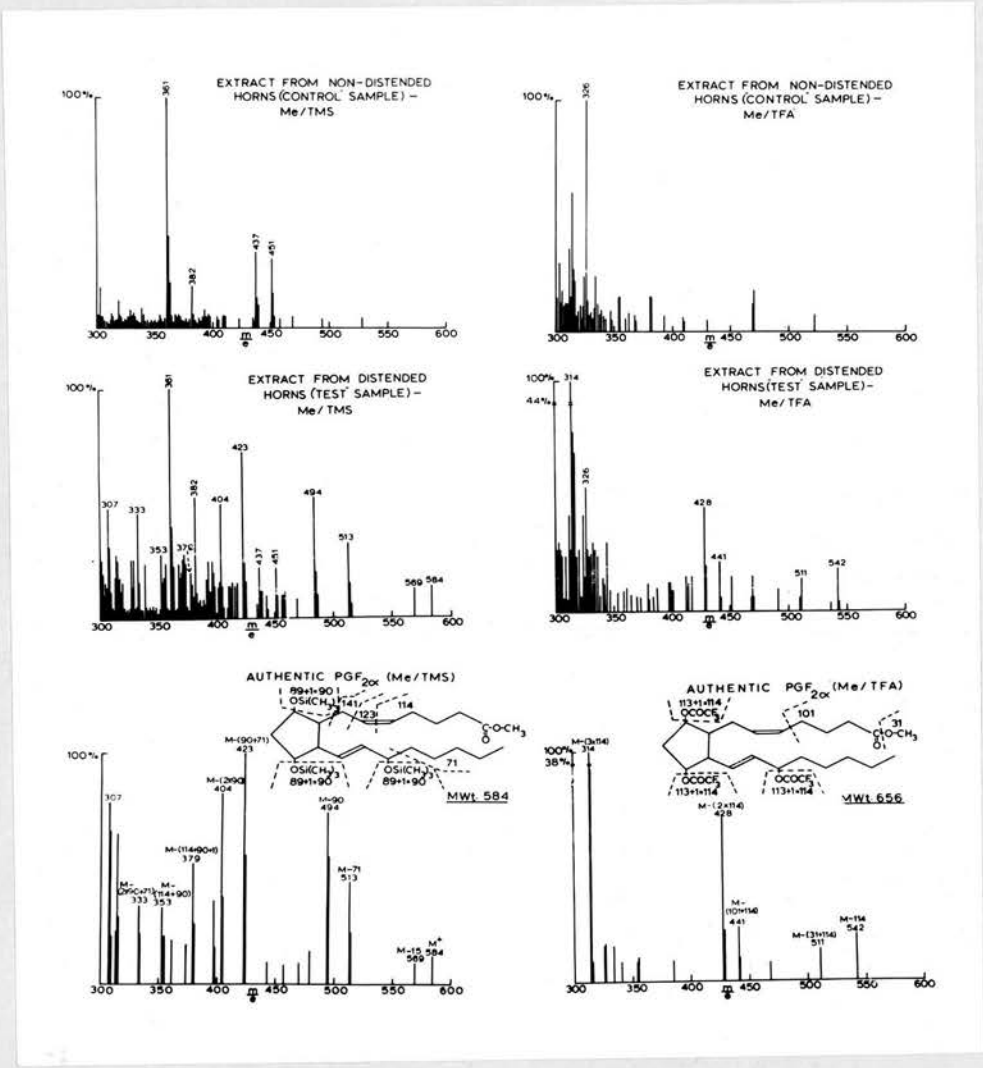

Fig. 8

Line diagrams of mass spectra ($^{m}/_{e}$ peaks greater than 300) of the Me/TMS and Me/TFA derivatives of "control" sample (from non-distended uterine horns), "test" sample (from distended uterine horns) and authentic prostaglandin F_2 Principal peaks characteristic of $F_2\alpha$ are indicated by horizontal numbering, other major peaks by vertical numbering (experiment 6). Ordinate: percentage relative intensity of peaks. Abscissa: $^{m}/_{e}$ value of peaks.

General Conclusion

In this series of experiments, an attempt has been made to assimilate *in vitro*, conditions which, *in vivo*, are known to promote the release of a uterine luteolytic substance. The cumulative evidence of solvent partition behaviour, column chromatography and pharmacological properties, the parallel bioassay results, and, in particular, the combined gas chromatography and mass spectrometry results has shown that distension of the guinea-pig uterus *in vitro* on days 3 or 4 of the oestrous cycle leads to the release of prostaglandin $F_2\alpha$. This substance is known to be luteolytic in guinea-pigs (Blatchley and Donovan, 1969). The result obtained here may account for the oestrous cycle shortening effect in guinea-pigs caused by distension of the uterus on days 2 to 4, which has been attributed to the premature release of a uterine luteolytic substance (Bland and Donovan 1966).

The mechanism by which distension of the uterus, *in vitro*, causes the release of prostaglandin $F_2\alpha$ has not been studied here or elsewhere. Section 5 indicates that it is not due to a simple breakdown of tissue with the release of stored prostaglandin. However, it may be due to a local breakdown of the endometrial tissue in contact with the polyethylene tubing with the release of both prostaglandin precursors and synthesising enzymes resulting in the production of prostaglandin $F_2\alpha$.

The contraceptive effect of intra-uterine devices in women has been attributed to an interaction between the device and the endometrium with resultant low grade inflammation and hyperaemia (Davies and Lesinski, 1970). Blatchley and Donovan (personal communication) have observed that the guinea-pig uterus into which beads have been placed has an enriched blood supply. This may indicate low grade inflammation with the resultant breakdown of cells and possibly synthesis and release of prostaglandin $F_2\alpha$. Prostaglandins are released during inflammatory conditions (Willis, 1969). This "cell breakdown theory" does not quite correlate with the

observations of Bland and Donovan (1968b). They found that the bead insertion on days 2, 4 and 8 only caused premature luteolysis, whilst bead insertion on days 5, 6, 7, 9 and 10 had no significant effect on the size of the corpora lutea at autopsy on day 12. (Bead insertion on day 3 was not studied, although Donovan and Traczyk (1962) have shown this to cause premature luteolysis also). However, close examination of the results on day 8 shows that the standard error of corpora lutea size in the control group on that day is small compared with the same standard errors in all other groups. A similar standard error would probably have made the result not significant. Therefore the significant result obtained on day 8 may be an artifact of statistical analysis. Blatchley and Donovan (1970) showed that bead insertion on days 5 to 8, the period of implantation, resulted in the production of large deciduomata. They speculate that the induction of deciduomata blocks the regression of the corpora lutea caused by the presence of foreign bodies in the uterus. The mechanism by which this is achieved was not discussed. It cannot be due to a release of the luteotrophic agent necessary for the maintenance of the corpora lutea during the early stages of pregnancy, which is antagonistic to the luteolytic agent, since this is produced by the placenta (Bland and Donovan, 1969b). However, Bland and Donovan (1968b) in their experiments reported that the premature luteal regression was not evident even in those animals treated on days 5 to 7 in which decidual tissue was absent. If their significant result obtained on day 8 is, in fact, an artifact, it may be that the distension of the guinea-pig uterus, by bead insertion, on days 2 to 4 only, causes premature regression of the corpora lutea, whilst distension after this in the cycle has no significant effect on the corpora luteal size at autopsy on day 12. This would correlate well with the "cell breakdown theory" as to the mechanism of action, especially as the endometrium, the surface in contact with the polyethylene tubing, is the apparent site of the uterine luteolytic hormone (Butcher, Chu and Melampey, 1962a, b).

SECTION 2

The Release of Prostaglandin $F_2\alpha$ from the Uterus of Guinea-pigs following Oestrogen Treatment

The treatment of guinea-pigs with oestrogen in the early- or mid-part of the oestrous cycle causes premature regression of the corpora lutea (Bland and Donovan, 1968a; Choudary and Greenwald, 1969). In the hysterectomised guinea-pig, oestrogen treatment has no such effect (Rowlands, 1962), whereas in the hemi-hysterectomised guinea-pig, oestrogen treatment causes regression of the corpora lutea only in the ovary adjacent to the retained horn (Bland and Donovan, 1970). These findings indicate that the luteolytic effect of oestrogen is mediated via the uterus, its local nature being compatible with the involvement of the uterine luteolytic hormone (luteolysin). It is possible that the premature release of this hormone from the uterus may account for the luteolytic effect of oestrogen treatment. Therefore, since the uterine luteolytic hormone may be prostaglandin $F_2\alpha$ (see section 1), the prostaglandin $F_2\alpha$ level in the uterine venous blood of guinea-pigs following oestrogen treatment has been estimated.

Methods

Treatment of Guinea-pigs and Collection of Uterine Venous Blood Samples (performed by F. R. Blatchley and B.T. Donovan).

Guinea-pigs were injected subcutaneously with 10 µg oestradiol benzoate/day from day 4 to day 6 of the cycle (day 1 = day of oestrus). On day 7 each was anaesthetised with pentobarbitone sodium and injected with 5000IU heparin, intravenously. The utero-ovarian vein on one side was exposed and blood collected from it into a cooled tube by means of a needle and silastic catheter for approximately 1 - 1½ hr. Centrifugation of the blood at 4°C was carried out as soon after collection as possible and the plasma stored at -20°C until extracted. In addition, five

guinea-pigs were hysterectomised on day 4, treated with 10 μg oestradiol benzoate/day from days 4-6, and blood collected from the intact utero-ovarian vein on day 7. Blood from untreated guinea-pigs was similarly collected on day 7 for control purposes.

Extraction of Plasma Samples

Plasma from 4-5 treated guinea-pigs was pooled in each experiment so as to give a volume of approximately 30 ml. Plasma samples from untreated guinea-pigs were pooled similarly. Each pooled sample was extracted by the solvent partition procedure as in section 1, experiment 2 (methods). The dry residue obtained was dissolved in 0.5 ml 30% ethyl acetate in benzene and subjected to silicic acid column chromatography as in section 1, experiment 5 (methods). Each fraction obtained from the columns was evaporated to dryness, the residue dissolved in 1 ml water and assayed on the rat fundal strip in the manner described in section 1, experiment 1 (methods). Fractions 1-5 were assayed against a standard solution of prostaglandin E_1 (100 ng/ml), fractions 6-8 against a similar solution of prostaglandin $F_2\alpha$.

Mass Spectrometry/Gas Chromatography - Where analysis by combined gas chromatography and mass spectrometry was performed, fraction 6 from the silicic acid column, following bioassay, was evaporated to dryness, the residue dissolved in methanol and transferred to a 0.5 ml stoppered tube. The methyl ester/trimethylsilyl ether (Me/TMS) was prepared, as in section 1, experiment 5 (methods). The Me/TMS derivative of authentic prostaglandin $F_2\alpha$ (500 ng) and of 5α, 7α-dihydroxy-11-oxo-tetranorprostanoic acid were prepared in a similar manner also. Ten microlitre quantities of these two standards were injected separately on to the gas chromatographic column. Their retention times were noted and mass spectra taken. A 10μl quantity of the Me/TMS derivative of each fraction 6 was injected on to the gas chromatographic column also. Mass spectra were taken of the effluent from the column at the previously noted retention times for the two standard compounds.

Recovery Experiments - Experiments were performed to estimate the percentage recovery of prostaglandin $F_2\alpha$ from the guinea-pig blood over a range of concentrations. Three guinea-pigs were stunned by a blow on the head, their necks incised and blood allowed to drain from the wound into a heparinised vessel. The amounts obtained varied between 10 and 20 ml. Sufficient prostaglandin $F_2\alpha$ was added to give concentrations of 10, 30 and 330 ng/ml. The blood was maintained at 37°C for one hour after which each sample was centrifuged and the red cells discarded. Each plasma sample was then solvent extracted and subjected to silicic acid column chromatography, as previously described. The fractions obtained from the columns were evaporated to dryness, the residues dissolved in 1.0 ml water and assayed on the rat fundal strip. Fractions 1-5 were assayed against prostaglandin E_1, fractions 6-8 against prostaglandin $F_2\alpha$.

Results

Prostaglandins of the "F" series are eluted from silicic acid columns by 80% ethyl acetate in benzene (fraction 6) (see section 1, experiment 5, results). In the recovery experiments, prostaglandin $F_2\alpha$ was eluted from columns by this fraction in every case. The average percentage recovery was 45.5% (range 40-50%) and was independent of the initial concentration of prostaglandin $F_2\alpha$ in the blood over the range tested.

The level of prostaglandin $F_2\alpha$ in the utero-ovarian blood of treated, non-treated and treated, hysteractomised guinea-pigs is shown in Table 8. These levels are based on the bioassay result of fraction 6 from the silicic acid columns and are corrected for a recovery of 45.5%.

Experiment No.	Level of $PGF_2\alpha$ in Utero-Ovarian Blood (ng/ml)	
	Oestrogen Treated	Untreated
1	19-25	<3.0
2	25-29	<3.8
3	11-13	<6.7
4	3.3	-
5	2.2-4.2	<5.2
6*	9-11	<2.2
7	4.2-6.2	<1.9
8*†	11-13	<1.9

* Gas chromatography and Mass Spectrometry performed in these experiments.

† Hysterectomised oestrogen-treated <1.8 ng $PGF_2\alpha$/ml

Table 8: Level of Prostaglandin $F_2\alpha$ (ng/ml) in Blood taken from the Utero-Ovarian Vein on Day 7 of the Oestrous Cycle of Oestrogen Treated and Control Guinea-pigs

In six out of eight experiments there was a greater amount of prostaglandin $F_2\alpha$ in the utero-ovarian blood of intact guinea-pigs pretreated with oestrogen than in non-treated control animals. The levels ranged from 4 to 29 ng/ml. No prostaglandin $F_2\alpha$ could be detected in the control groups, nor in the oestrogen treated, hysterectomised group (< 1.8 ng/ml).

Results of Gas Chromatography/Mass Spectrometry

Analysis by combined gas chromatography and mass spectrometry was performed in two of the eight experiments. The Me/TMS derivative of authentic prostaglandin $F_2\alpha$ had a retention time on gas chromatography of 19.3 minutes. Mass spectra taken at this retention time of the effluent from the gas chromatographic column of the Me/TMS derivative of the various samples analysed in one experiment are summarised in the line diagrams in Figure 9. As noted previously, the characteristic $^m/_e$ peaks of values above 300 of the Me/TMS derivative of authentic prostaglandin $F_2\alpha$ occur at

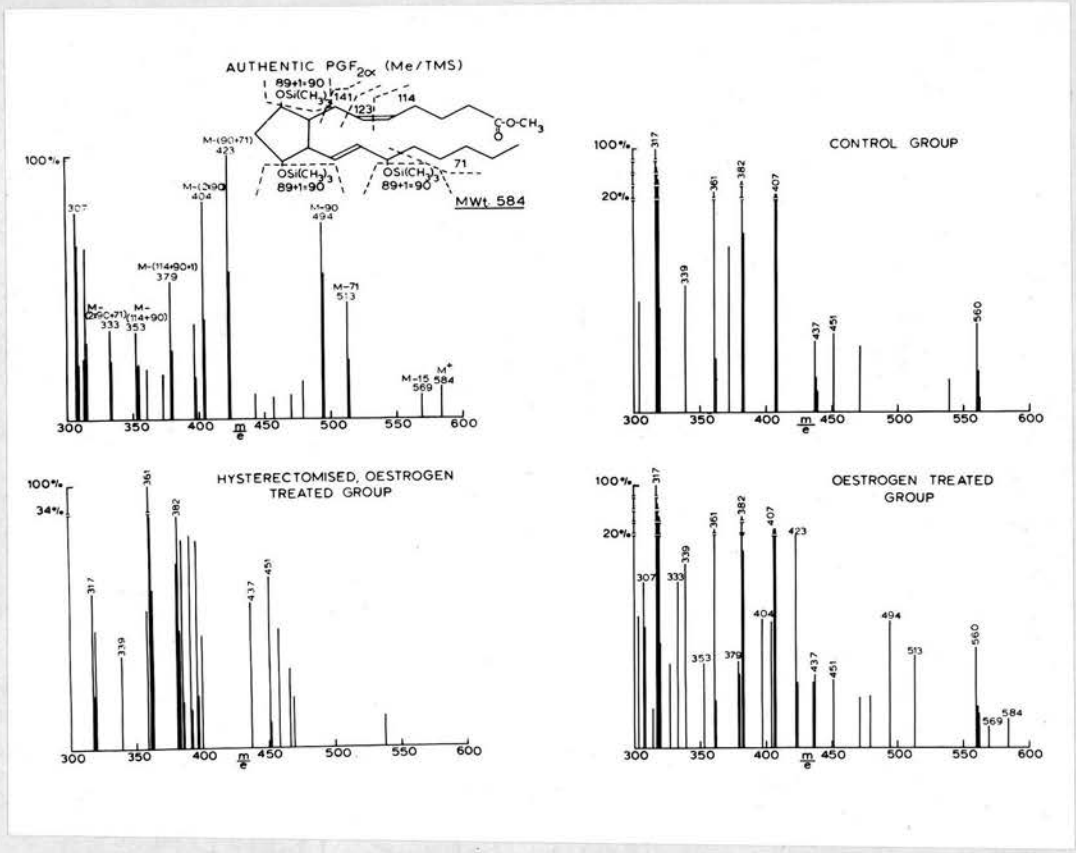

Fig. 9

Line diagrams of mass spectra ($^{m}/e$ peaks greater than 300) of the Me/TMS derivative of material extracted from the utero-ovarian venous blood of intact guinea-pigs treated with oestrogen, hysterectomised guinea-pigs treated with oestrogen, and intact, non-treated control animals, and of authentic prostaglandin $F_{2\alpha}$. Retention time on gas chromatography 19.3 minutes. Principal peaks characteristic of $F_{2\alpha}$ are indicated by horizontal numbering, other major peaks by vertical numbering. Ordinate: percentage relative intensity of peaks. Abscissa: $^{m}/e$ value of peaks.

307, 333, 353, 379, 404, 423, 494, 513, 569 and 584. These peaks are present in the mass spectrum of the Me/TMS derivative of the extracted utero-ovarian venous blood taken from the guinea-pigs treated with oestrogen, but not in the mass spectra of the same derivative of similar blood taken from oestrogen treated, hysterectomised animals, nor non-treated, intact animals. Other peaks occurring in the spectra are due to common impurities.

A duplicate result was obtained in the other experiment where gas chromatography and mass spectrometry was performed. This evidence shows conclusively that prostaglandin $F_{2\alpha}$ is present in the utero-ovarian blood of guinea-pigs pre-treated with oestrogen but cannot be detected in similar blood of pre-treated, hysterectomised guinea-pigs, nor in non-treated intact guinea pigs.

The main urinary metabolite of prostaglandin $F_{2\alpha}$ (5α, 7α - dihydroxy-11-oxo-tetranorprostanoic acid) is eluted from the silicic acid columns by 80% ethyl acetate in benzene (fraction 6) (Los, M., unpublished observations). The Me/TMS derivative of this metabolite on gas chromatography had a retention time of 5.3 minutes. Its mass spectrum had characteristic peaks of $^{m}/e$ values above 200 at 217, 241, 254, 278, 281, 353, 368, 427 and 443. The molecular ion ($^{m}/e$ 458) was not seen.

Mass spectra taken at the retention time of 5.3 minutes of the effluent from the gas chromatographic column of the Me/TMS derivative of fraction 6 from the silicic acid columns of the extracted utero-ovarian venous blood taken from treated and non-treated, intact guinea-pigs did not contain any of these peaks, except the one at $^{m}/e$ 217 (Figure 10). On this evidence it can be concluded that the main guinea-pig metabolite of prostaglandin $F_{2\alpha}$ was not present in amounts that could be detected in the utero-ovarian venous blood of these guinea-pigs. (Utero-ovarian venous blood taken from the oestrogen treated, hysterectomised group of guinea-pigs was not analysed for the presence of this metabolite).

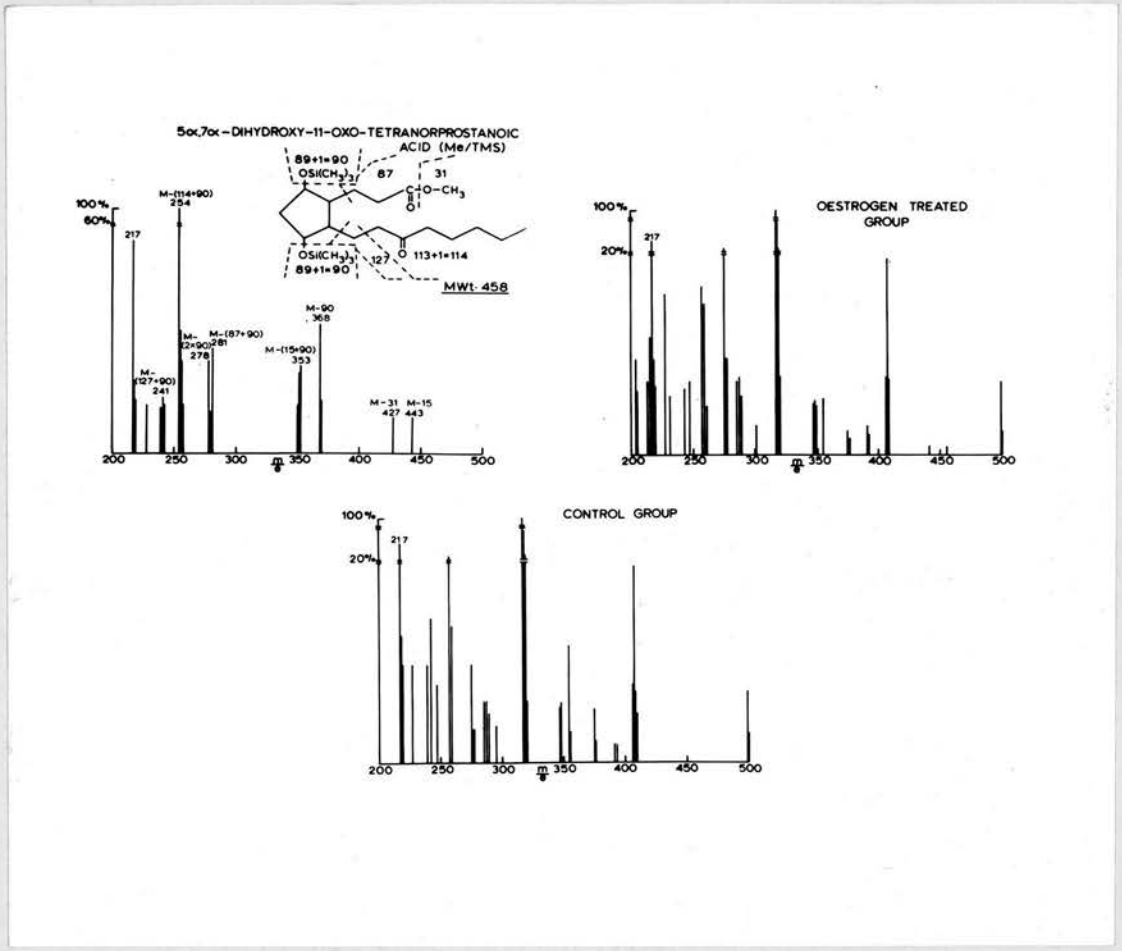

Fig. 10

Line diagrams ($^{m}/e$ peaks greater than 200) of the Me/TMS derivative of material extracted from the utero-ovarian venous blood of oestrogen treated and non-treated (control) guinea-pigs, and of authentic 5α, 7α -dihydroxy-11-oxo-tetranorprostanoic acid. Retention time on gas chromatography, 5.3 minutes. Peaks characteristic of the authentic compound are indicated by horizontal numbering. Ordinate: percentage relative intensity of peaks. Abscissa: $^{m}/e$ value of peak.

In addition, on the basis of gas chromatographic evidence, prostaglandin $F_1\alpha$ could not be detected in those samples analysed. Furthermore, on the basis of the bioassay results only, no prostaglandins of the 'E' series were detected in any of the blood samples analysed (< 0.5 ng prostaglandin E_1/ml - this value is uncorrected for recovery.)

Conclusion

The treatment of guinea-pigs with oestrogen during the early- or mid-part of the oestrous cycle causes early regression of the corpora lutea due probably to the premature release of a uterine luteolytic substance (Bland and Donovan, 1970). The experiments performed in this section have shown prostaglandin $F_2\alpha$ to be present in the utero-ovarian venous blood of guinea-pigs receiving oestrogen treatment whereas no prostaglandin $F_2\alpha$ could be detected in similar blood taken from non-treated animals. In two experiments, combined gas chromatography and mass spectrometry confirmed the identity of the prostaglandin $F_2\alpha$. Utero-ovarian venous blood taken from hysterectomised, oestrogen treated guinea-pigs did not contain any detectable amounts of prostaglandin $F_2\alpha$. This shows that the prostaglandin $F_2\alpha$ present in the utero-ovarian venous blood of intact, oestrogen treated guinea-pigs originated in the uterus and not the ovary. Neither prostaglandin $F_1\alpha$ nor the main guinea-pig metabolite of prostaglandin $F_2\alpha$ (5α, 7α-dihydroxy-11-oxo-tetranorprostanoic acid) could be detected in samples analysed for their presence.

The release of prostaglandin $F_2\alpha$, a potent luteolytic substance, from the uterus of oestrogen treated guinea-pigs provides a possible explanation for the luteolytic effect of oestrogen treatment. Its presence in the utero-ovarian venous blood correlates well with the uterine venous drainage being involved in the luteolytic effect of the uterus on the ovary. Furthermore, the absence of prostaglandin $F_2\alpha$ in the utero-ovarian venous blood of oestrogen treated, hysterectomised guinea-

pigs is in agreement with the lack of luteolytic activity of oestrogen treatment in such animals. Consequently the findings in this section not only provide a possible explanation for the luteolytic effect of oestrogen treatment in guinea-pigs, but provide also further evidence in favour of the hypothesis that the uterine luteolytic hormone (luteolysin) is prostaglandin $F_2\alpha$.

SECTION 3

Levels of Prostaglandins in the Uterine Venous Blood of Sheep and Guinea-pig during the Oestrous Cycle

The previous two sections have shown that the guinea-pig uterus can produce prostaglandin $F_2\alpha$, a substance known to be luteolytic, under conditions associated with the premature release of the uterine luteolytic hormone. The pathway for the luteolytic action of the uterus on the ovary appears to be provided by the local vascular system (Oxenreider and Day, 1967; Bland and Donovan, 1969; Kiracofe et al., 1963, 1966). Since the luteolytic hormone appears to be released from the uterus in greater amounts towards the end of the oestrous cycle, the levels of prostaglandins in the uterine venous blood of sheep and guinea-pig during the cycle have been studied.

Methods

Collection of Blood from Sheep

Mature Cheviot ewes were checked for oestrus by pairing with a vasectomised ram once or twice daily. The first day of acceptance was taken as day 1 of the cycle. Laparotomies were performed on selected days of the cycle and 40 ml samples of uterine venous blood withdrawn. A 25 ml sample and an 80 ml sample were taken, however, from ewes on day 2 and 15 respectively. Anaesthesia was induced and maintained with halothane and nitrous oxide. No ewe was operated on more than twice, and each animal was killed several days after the final operation. An autopsy was performed to ensure that the uterus and ovaries were of normal appearance. Each sample of blood obtained was centrifuged, the plasma withdrawn and extracted.

Collection of Blood from Guinea-pigs (performed by F. R. Blatchley and B.T. Donovan)

Blood was collected from the utero-ovarian vein of normal guinea-pigs on selected days of the oestrous cycle in the same manner as described in section 2 (methods).

Centifrugation of the blood at 4°C was carried out as soon after collection as possible and the plasma stored at -20°C. After thawing the plasma from five guinea-pigs on each of the days selected was pooled so as to give volumes of 30-45 ml for extraction.

Extraction of Plasma Samples

Plasma samples obtained from both sheep and guinea-pigs were treated by the solvent extraction procedure for the isolation of polar acidic lipids, as in section 1, experiment 2 (methods). The dry residues obtained were dissolved in 0.5 ml 30% ethyl acetate in toluene and subjected to silicic acid column chromatography, as in section 1, experiment 5 (methods).

Silicic Acid Column Chromatography of Sheep Uterine Venous Blood Extracts

Columns of 4.2 g silicic acid (Sigma SIL-R, 100 mesh; or, Bio-Rad Labs BIO SIL A, 100-200 mesh control no. 6910 or 6583A) were used. Toluene was substituted for benzene in the eluant mixture. The percentage of ethyl acetate in and the volume of each fraction were the same as in Table 5 (see section 1) except that fractions 3, 4 and 5 contained 65% ethyl acetate, not 60%. This change had no effect on the elution of prostaglandins from columns when prepared using Sigma SIL-R, 100 mesh or Bio-Rad labs BIO SIL A, 100-200 mesh, control no. 6583A silicic acid. Prostaglandins of the "E" series are eluted by 65% ethyl acetate in toluene (fraction 4) and prostaglandins of the "F" series by 80% ethyl acetate in toluene (fraction 6) (cf. section 1, experiment 5, results). However when columns were built using Bio-Rad Labs BIO SIL A, 100-200 mesh, control no. 6910 silicic acid, this solvent system proved unsatisfactory. By using authentic prostaglandins (1.5 μg each of PGE_1 and of $PGF_{2\alpha}$), it was found that a small percentage of methanol had to be added to each fraction to aid elution of the prostaglandins from these columns (see results part of this section). The percentages of solvent used in the volumes of each fraction are shown in Table 9.

Fraction	Volume (ml)	Percentage		
		Methanol	Ethyl Acetate	Toluene
1	50	1	29	70
2	50	2.5	37.5	60
3	20	4	61	35
4	150	4	61	35
5	20	4	61	35
6	200	5.5	74.5	20
7	50	5.5	94.5	-
8	50	100	-	-

Table 9: Percentages of Eluant in and Volume of Each Fraction used to Elute Columns of Silicic Acid, Bio-Rad Labs BIO SIL A, 100-200 mesh, control no. 6910.

Another problem was the possible overloading of the columns with material. This became apparent when the initial blood sample was slightly haemolysed. It was overcome by using larger columns (10 g) and twice as much eluant in each fraction.

The sheep uterine venous blood extracts were loaded on to the columns in a dropwise manner using a Pasteur pipette, and the columns eluted by the appropriate fractions of eluant. The fractions obtained from the columns were evaporated to dryness, the residues dissolved in 1 ml water and assayed on the rat fundal strip against a standard solution of prostaglandin $F_{2\alpha}$ (100 ng/ml).

Silicic Acid Column Chromatography of Guinea-pig Uterine Venous Blood Extracts:

Columns were prepared using 4.2 g silicic acid (Bio-Rad Labs, BIO SIL A, 100-200 mesh, control no. 6583A) and eluted by increasing concentrations of ethyl acetate in toluene (with no methanol added) as used for the sheep extracts. Overloading of the columns was not a problem, therefore larger columns were not required. The fractions obtained from the columns were evaporated to dryness, under reduced pressure, the residues dissolved in 1 ml water and assayed on the rat fundal strip. Fractions

1-5 were assayed against a standard solution of prostaglandin E_2 (100 ng/ml) whilst fractions 6-8 were assayed against a similar solution of prostaglandin $F_{2\alpha}$.

Gas Chromatography/Mass Spectrometry:

The fractions in which prostaglandin $F_{2\alpha}$ appeared, or would have appeared if present, following silicic acid chromatography of both the sheep and guinea-pig uterine venous blood extracts, were subjected to analysis by combined gas chromatography and mass spectrometry. After bio-assay, the appropriate fractions were evaporated to dryness, in vacuo, the residues dissolved in methanol and transferred to 0.5 ml stoppered tubes. The methyl ester/trimethylsilyl ether (Me/TMS) derivative of each was prepared as in section 1, experiment 5 (methods), by adding 15 μl of BSTFA at the final stage in the case of the sheep extracts and 25 μl in the case of the guinea-pig extracts.

The Me/TMS derivative of authentic prostaglandin $F_{2\alpha}$ (500 and 250 ng) was also prepared. Ten microlitre quantities were injected on to the gas chromatographic column and the retention times noted. A mass spectrum was recorded at this time.

The Me/TMS derivative of the appropriate fractions from the silicic columns were then injected on to the gas chromatographic column. Mass spectra were taken of the effluents from the column at the previously noted retention time for the Me/TMS derivative of authentic prostaglandin $F_{2\alpha}$.

In addition, the methyl ester/trifluoroacetate (Me/TFA) of one guinea-pig uterine venous blood extract was prepared by the method described in section 1, experiment 6 (methods). The Me/TFA derivative of authentic prostaglandin $F_{2\alpha}$ was also prepared and its retention time on gas chromatography noted. A mass spectrum was recorded at this time. The Me/TFA derivative of the guinea-pig extract was then injected on to the gas chromatographic column and a mass spectrum taken of the effluent at the retention time for the Me/TFA derivative of authentic prostaglandin $F_{2\alpha}$.

In two instances, fraction 4 following silicic acid column chromatography of the

guinea-pig uterine venous extracts was biologically active. Consequently these fractions were analysed for the presence of prostaglandins of the "E" series, by one or both of the following methods:-

Method 1: The sample was evaporated to dryness, in vacuo, dissolved in methanol and transferred to a 0.5 ml stoppered tube. The methyl ester was prepared as in section 1, experiment 5 (methods), excess diazomethane solution being vaporised to leave a dry residue. The methoxime was then prepared by the addition of 150μl of a freshly prepared solution of 1% methoxyamine hydrochloride in pyridine. After a twelve hour reaction period the sample was evaporated to dryness, in vacuo. The trimethylsilyl ether was formed subsequently by the addition of 25μl BSTFA.

The methyl ester/methoxime/trimethysilyl ether (Me/MO/TMS) of authentic prostaglandin E_1 (1μg) and prostaglandin E_2 (1μg) were prepared in a similar manner. Each was injected on to the gas chromatographic column. Methoximes exist as two stereoisomers which are separated by gas chromatography. Thus two peaks were obtained, the retention time for each peak being noted for both compounds. A mass spectrum was recorded at these times. The Me/MO/TMS derivative of each fraction 4 was then injected on to the gas chromatographic column. Mass spectra of the effluent from the column were recorded at the previously noted retention times for both stereoisomers of the Me/MO/TMS derivative of prostaglandins E_1 and E_2.

Method 2: The sample was first evaporated to dryness, then 0.5 ml of 0.1 N potassium hydroxide in methanol was added and allowed to stand at 37°C for 30 minutes. Under these conditions the prostaglandin of the "E" series undergoes dehydration, with the formation of the corresponding prostaglandin of the "A" series. This in turn is isomerised into the more stable prostaglandin B compound (see Figure 11).

After the reaction period, 20 ml of water was added to the reaction flask, the acidity adjusted to pH4 with hydrochloric acid, and partitioned twice with 40 ml of

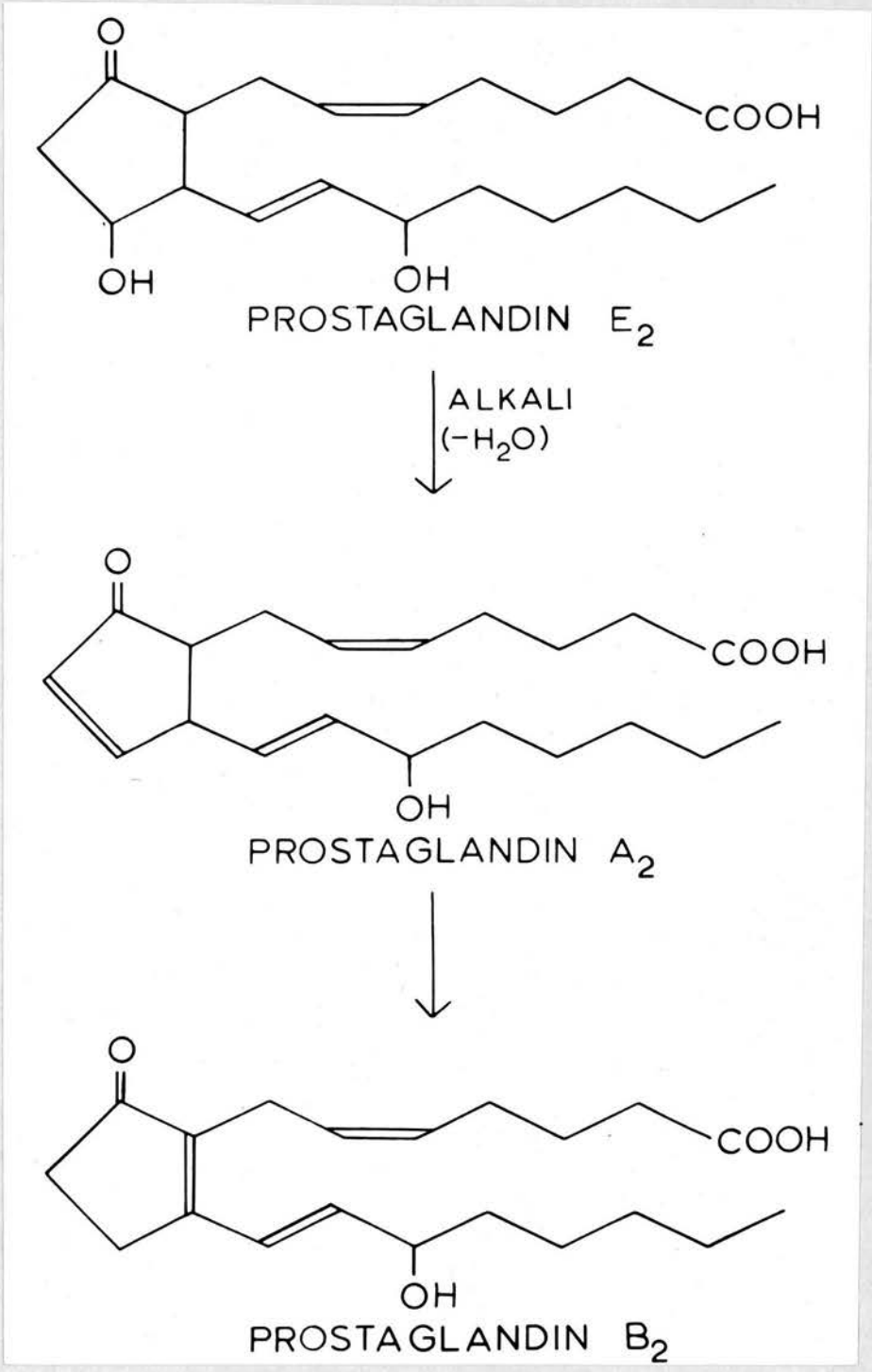

Fig. 11

The chemical conversion of prostaglandin E_2 to prostaglandin B_2

ethyl acetate. The two ethyl acetate fractions were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 0.2 ml methanol and spotted on to a pre-prepared thin layer plate (20 cm x 5 cm) coated with silica gel to a thickness of 0.25 mm (Merck). An identical plate spotted with 10 μg of pros - taglandin B_2 acted as a marker. Both plates were developed simultaneously using a solvent system of the following composition:- Toluene: 1, 4-Dioxan: Acetic Acid, 25 : 15 : 0.5. A 10 cm run was allowed.

Following chromatography the marker plate was visualised by spraying with 10% phosphomolybdic acid in ethanol and heating at 110°C for five minutes. The R_F value of prostaglandin B_2 was noted. A zone, 1.5 cm wide with its midline corresponding to the R_F of prostaglandin B_2, was scraped off the test plate. The silica gel was eluted by shaking with 5 ml methanol. The resultant suspension was centrifuged and the methanol withdrawn. This was repeated with another 5 ml of methanol. The two methanol fractions were then combined, reduced in volume by low pressure evaporation, transferred to a 0.5 ml stoppered tube and finally evaporated to dryness. The Me/TMS derivative of the residue was then formed as in section 1, experiment 5 (methods).

The Me/TMS derivative each of 1 μg of prostaglandins B_1 and B_2 was also prepared. These were injected on to the gas chromatographic column and their retention times noted, (column temperature = 195°C). A mass spectrum of each was recorded. The Me/TMS derivative of the extracted material was then injected on to the column and mass spectra recorded at the noted retention times.

Recovery Experiments

a) From Sheep Blood: During some of the operations on the ewes, a peripheral sample of blood (40-45 ml) was taken from the jugular vein. To this was added sufficient prostaglandin $F_{2\alpha}$ to produce a final concentration of 10 ng/ml. To one

jugular venous sample from a sheep on day 15, prostaglandin was not added. These samples were treated exactly as described above. They provided estimates as to the percentage recovery of prostaglandin $F_{2}\alpha$ from sheep's blood, and the reliability of the extraction and isolation process as a whole.

b) From Guinea-pig Blood:

i) The percentage recovery of prostaglandin $F_{2}\alpha$ from guinea-pig blood was determined in section 2. The result obtained there (45.5%) has been used in these experiments to calculate levels of prostaglandin $F_{2}\alpha$ in uterine venous blood of guinea-pigs.

ii) The percentage recovery of prostaglandin E_{2} from guinea-pig blood was determined in a similar manner to that of prostaglandin $F_{2}\alpha$ described in section 2. Three guinea-pigs were killed, their necks incised and blood allowed to drain from the wounds into heparinised vessels. Quantities of 10 to 16 ml were obtained and sufficient prostaglandin E_{2} added to produce concentrations of 10, 30 and 50 ng/ml. After standing for one hour, each blood sample was centrifuged and the red cells discarded. The plasma was treated as described earlier. The percentage recovery of prostaglandin E_{2} from the guinea-pig blood was calculated from the bioassay results obtained.

Results

1) Column Chromatography of the Authentic Prostaglandins Using Bio-Rad Labs BIO SIL A 100-200 mesh control no. 6910 silicic acid

Table 10 summarises the bioassay results obtained. Prostaglandin E_{1} was eluted in fraction 4 whereas prostaglandin $F_{2}\alpha$ was eluted in Fraction 6.

Fraction	Volume (ml)	Percentage			Bioassay Result on Rat Fundal Strip
		Methanol	Ethyl Acetate	Toluene	
1	50	1	29	70	< 50 ng PGE_1
2	50	2.5	37.5	60	< 50 ng PGE_1
3	20	4	61	35	< 50 ng PGE_1
4	150	4	61	35	1 µg PGE_1
5	20	4	61	35	< 50 ng PGE_1
6	200	5.5	74.5	20	1 µg $PGF_{2\alpha}$
7	50	5.5	94.5	-	< 50 ng $PGF_{2\alpha}$
8	50	100	-	-	100 ng $PGF_{2\alpha}$

Table 10: Results of the Column Chromatography of Authentic Prostaglandins Using Bio-Rad Labs BIO SIL A, 100-200 mesh Control no. 6910 Silicic Acid. A Small Percentage of Methanol has been added to each Fraction to aid Elution

Prostaglandins subjected to column chromatography using this particular batch of silicic acid without the addition of methanol to each fraction were not eluted from the columns until the final methanol stage and separation of the different series of prostaglandins was not achieved. Consequently by adding a small percentage (1-5.5%) of methanol, the polarity of solvents in each fraction was increased sufficiently to elute prostaglandins from the column and to achieve a separation.

2) Sheep Blood Extracts

The oestrous cycle in all nine ewes was 17 days in length. The operational procedure had no effect on the recurrence of oestrus although in two ewes behavioural oestrus was either weak or absent. This was probably due to the close proximity of the end of the breeding season. At autopsy the uterine horns and ovaries appeared normal. Consequently there was no reason to believe that the collecting of uterine and peripheral blood samples had affected the sheep in any way.

i) Recovery Experiments: The last column of Table 11 shows the percentage recovery of added prostaglandin $F_2\alpha$ from jugular venous blood. Gas chromatography and mass spectrometry confirmed that the material recovered was prostaglandin $F_2\alpha$.

Amount added (ng)	Amount Recovered (ng)	% Recovery
450	200	44.4
450	203	45.0
400	210	52.5
440	235	53.3
400	170	42.5
400	170	42.5
	Average % Recovery	46.7

Table 11: Percentage Recovery of Prostaglandin $F_2\alpha$ from the Peripheral Venous Blood of Sheep at a Concentration of 10 ng/ml

The average percentage recovery was 46.7% (range 42.5% to 53.3%). No prostaglandin $F_2\alpha$ (<2.9 ng/ml) could be detected in the peripheral venous blood, taken from a ewe on day 15, to which prostaglandin $F_2\alpha$ had not been added.

ii) Levels of Prostaglandin $F_2\alpha$ in the Uterine Venous Blood of Sheep:

The estimated levels of prostaglandin $F_2\alpha$ in the uterine venous blood taken from the ewes during the oestrous cycle and corrected for a 46.7% recovery are shown in the last column of Table 12. These figures are based on the combined evidence of bioassay, gas chromatography and mass spectrometry.

Ewe No.	Day of Cycle	Bioassay Result (Total Activity Extracted ≡ ng $PGF_{2\alpha}$)	Mass Spectrometry Result	Estimated Level of $PGF_{2\alpha}$ in Uterine Venous Blood (ng/ml)
2	2	< 35	Possible Trace	< 3.0
8	4	< 50	n.d.	< 2.7
2	7	< 50	n.d.	< 1.9
10	7	50-75	n.d.	< 2.7
10	9	< 50	n.d.	< 2.7
3	10	180-200	Possible Trace	< 2.7
12	11	< 50	n.d.	< 2.7
5	13	< 50	n.d.	< 2.7
3	14	135-165	$PGF_{2\alpha}$ present	7.2-8.8
6	15	115-135	---	6.1-7.2
11	15	120-130	$PGF_{2\alpha}$ present	3.2-3.5
4	16(a)	90-120	$PGF_{2\alpha}$ present	4.8-6.4
4	16(b)	60-80	---	3.2-4.3

n.d. means no $PGF_{2\alpha}$ detected.
--- means $PGF_{2\alpha}$ peaks masked by interfering substances.
(a) and (b) are from different cycles.

Table 12: Level of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the Uterine Venous Blood of Sheep during the Oestrous Cycle

From day 2 to day 13, no prostaglandin $F_{2\alpha}$ (<3 ng/ml) could be identified in uterine venous blood, whereas on days 14, 15 and 16 the estimated levels ranged from 3.4 to 8.0 ng/ml. In ewe 10 (day 7) and ewe 3 (day 10) there was smooth muscle stimulating activity in the appropriate fraction from the silicic acid column, but this was not due to prostaglandin $F_{2\alpha}$ as shown by the results of gas chromatography and mass spectrometry. However, a possible trace amount may have been present in ewe 3 (day 10) and in ewe 2 (day 2). This was based solely on the existence in the mass spectra of very small peaks at $^{m}/e$ values of 494 and 513. These two peaks are characteristic of the Me/TMS derivative of prostaglandin $F_{2\alpha}$. The levels they

represented were probably less than 15 ng. Approximately 50-100 ng of prostaglandin $F_2\alpha$ is needed for conclusive mass spectral evidence to be obtained when interfering substances are present in extracts of biological material. The amount required depends upon the purity of the final extract. This was evident in ewe 6 (day 15) and ewe 4 (day 16b). The final extract obtained contained unusually large amounts of interfering substances. Thus conclusive mass spectral confirmation could not be obtained.

Examples of the evidence provided by gas chromatography and mass spectrometry are shown in Figure 12. The retention time of the Me/TMS derivative of prostaglandin $F_2\alpha$ was 19.5 minutes. The line diagrams represent, for each sample, mass spectra taken at this time of the effluent from the gas chromatographic column. Any peaks characteristic of the Me/TMS derivative of authentic prostaglandin $F_2\alpha$ of $^m/e$ value below 400 were lost in the background noise. Therefore the identification of prostaglandin $F_2\alpha$ in the samples was based on the presence in the mass spectrum of the six peaks at $^m/e$ values of 404, 423, 494, 513, 569 and 584. These were present, in the correct ratios, in the trace obtained from ewe 3 (day 14), but were not present in the trace obtained from ewe 2 (day 7). The uterine venous blood extracts from both gave five other peaks which were not found in authentic prostaglandin $F_2\alpha$. These are due to a common impurity. This evidence shows that prostaglandin $F_2\alpha$ is present in the uterine venous blood taken on day 14, but not in the uterine venous blood taken on day 7. Similar conclusions were drawn regarding other samples.

3) Guinea-pig Blood Extracts:

i) Recovery Experiment: The percentage recoveries of prostaglandin E_2 from guinea-pig blood at concentrations of 10, 30 and 50 ng/ml were 42.5%, 46.4% and 47.5% respectively, the average recovery being 45.5%. These figures are based solely on the bioassay result obtained on the rat fundal strip following solvent

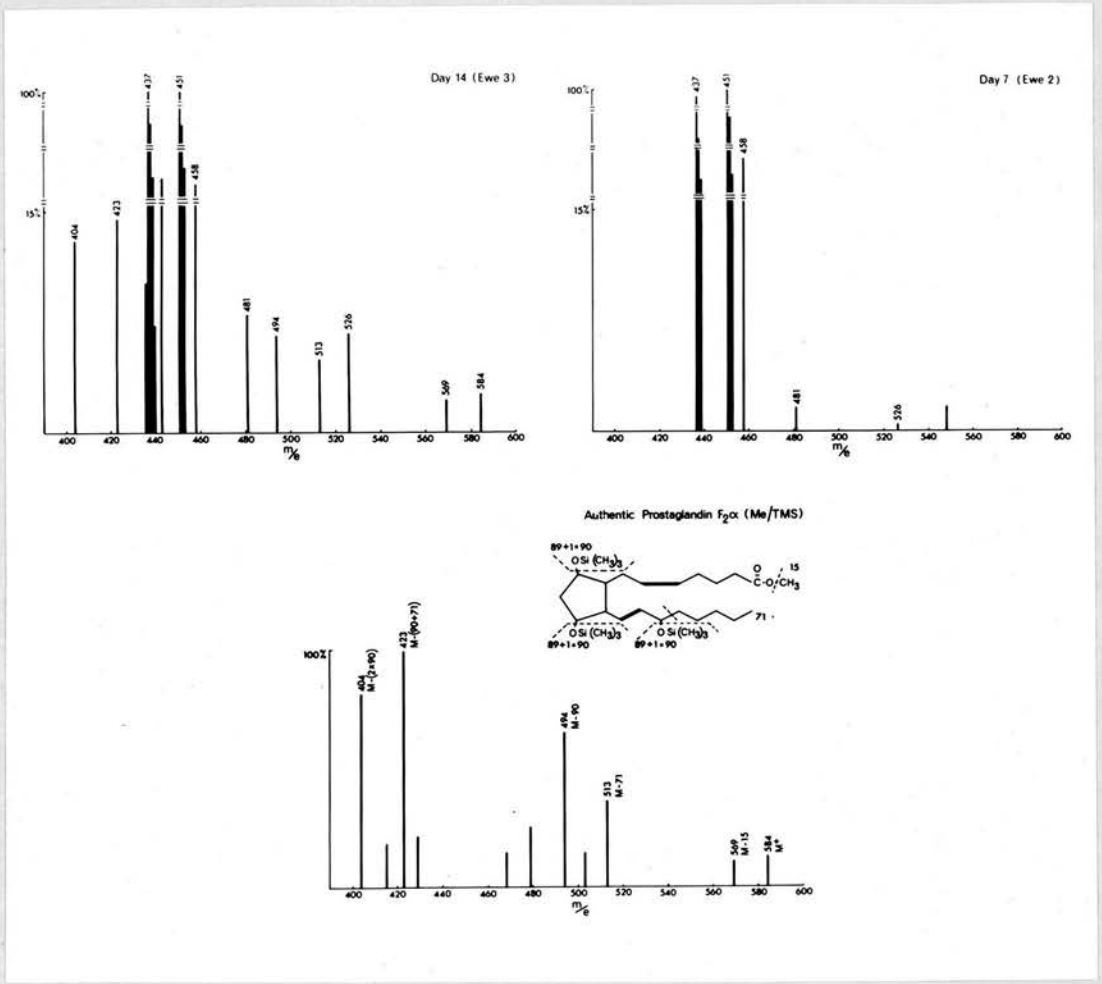

Fig. 12

Line diagrams of mass spectra ($^{m}/_{e}$ peaks greater than 400) of the Me/TMS derivative of material extracted from uterine venous blood samples taken from sheep on day 14 and day 7, and of authentic prostaglandin $F_{2\alpha}$.
Ordinate: percentage relative intensity of peaks.
Abscissa: $^{m}/_{e}$ value of peaks.

extraction and silicic acid column chromatography of the blood samples. The recovery of prostaglandin $F_{2}\alpha$ from guinea-pig blood was 45.5% also (see section 2, results).

ii) Levels of Prostaglandins in the Utero-ovarian Venous Blood of Guinea-pigs. The estimated levels of prostaglandin $F_{2}\alpha$ and E_{2} in the utero-ovarian venous blood of guinea-pigs during the oestrous cycle are shown in Table 13. These figures are based on the bioassay results and are corrected for recoveries of 45.5% for prostaglandin $F_{2}\alpha$ and for prostaglandin E_{2}. The level of prostaglandin $F_{2}\alpha$ on day 7 has been taken from the results obtained in section 2 for the non-treated, intact groups of guinea-pigs and represents the average value recorded. No figure for the level of prostaglandin E_{2} on day 7 has been included, since in those experiments, the fraction from the silicic acid columns in which prostaglandins of the "E" series would appear, if present, was assayed in terms of prostaglandin E_{1}. However, no prostaglandin E-like activity was detected.

Day of Cycle	Level of $PGF_{2}\alpha$ (ng/ml)	Level of PGE_{2} (ng/ml)
3	14.1	< 0.74
7	< 3.3	-
9	5.2	< 0.80
10	3.5	< 0.77
11	17.2	< 0.82
12	16.2	< 0.80
13	15.7	1.10
14	16.0	5.40
15	60.7	54.9

Table 13: Levels of Prostaglandin $F_{2}\alpha$ ($PGF_{2}\alpha$) and Prostaglandin E_{2} (PGE_{2}) In the Utero-ovarian Venous Blood of Guinea-pigs during the Oestrous Cycle. Blood from 5 Guinea-pigs was pooled for Each Day

The level of prostaglandin $F_{2}\alpha$ on day 3 was 14.1 ng/ml. This has fallen to below detectable limits (< 3.3 ng/ml) on day 7. On days 9 and 10, low levels (5.2

and 3.5 ng/ml) were present. The levels subsequently rose to values between 15.7 and 17.2 ng/ml on days 11, 12, 13 and 14. A large rise then occurred to a level of 60.7 ng/ml on day 15. After this peak, the level of prostaglandin $F_{2\alpha}$ must have soon started to decline as the level on day 3 reflected.

Prostaglandin E_2 was not detected in the utero-ovarian venous blood of guinea-pigs examined on days between 3 and 12. A detectable level, 1.1 ng/ml, was present on day 13 which rose to 5.4 ng/ml on day 14. The level then increased sharply to 54.9 ng/ml on day 15. A fall then set in as the level on day 3 reflected.

Identification of the Prostaglandins

a) Prostaglandin $F_{2\alpha}$: Gas chromatography and mass spectrometry provided conclusive evidence for the presence of prostaglandin $F_{2\alpha}$ in several of the guinea-pig blood samples. The Me/TMS and Me/TFA derivatives of authentic prostaglandin $F_{2\alpha}$ on gas chromatography had retention times of 16.6 and 4.9 minutes, respectively. Mass spectra were taken at these times of the column effluent of the Me/TMS and Me/TFA derivatives of material extracted from blood taken from day 15 guinea-pigs (see Figure 13). The spectrum of the Me/TMS derivative contains significant peaks at $^{m}/e$ values of 307, 333, 353, 379, 404, 423, 494, 513, 569 and 584, the peaks typical of those produced by the Me/TMS derivative of authentic prostaglandin $F_{2\alpha}$. The ratio between peak heights is approximately the same also. Similarly the mass spectra of the Me/TFA derivative of extracted material and authentic prostaglandin $F_{2\alpha}$ have peaks in common at $^{m}/e$ values of 314, 428, 441, 511 and 542, again in approximately the same ratios. This confirms that prostaglandin $F_{2\alpha}$ was present in the utero-ovarian venous blood of guinea-pigs taken on day 15 of the cycle. Similar evidence, based on a comparison of the mass spectra of the Me/TMS derivatives, confirmed the presence of prostaglandin $F_{2\alpha}$ in blood taken from guinea-pigs on days 13 or 14 of the cycle.

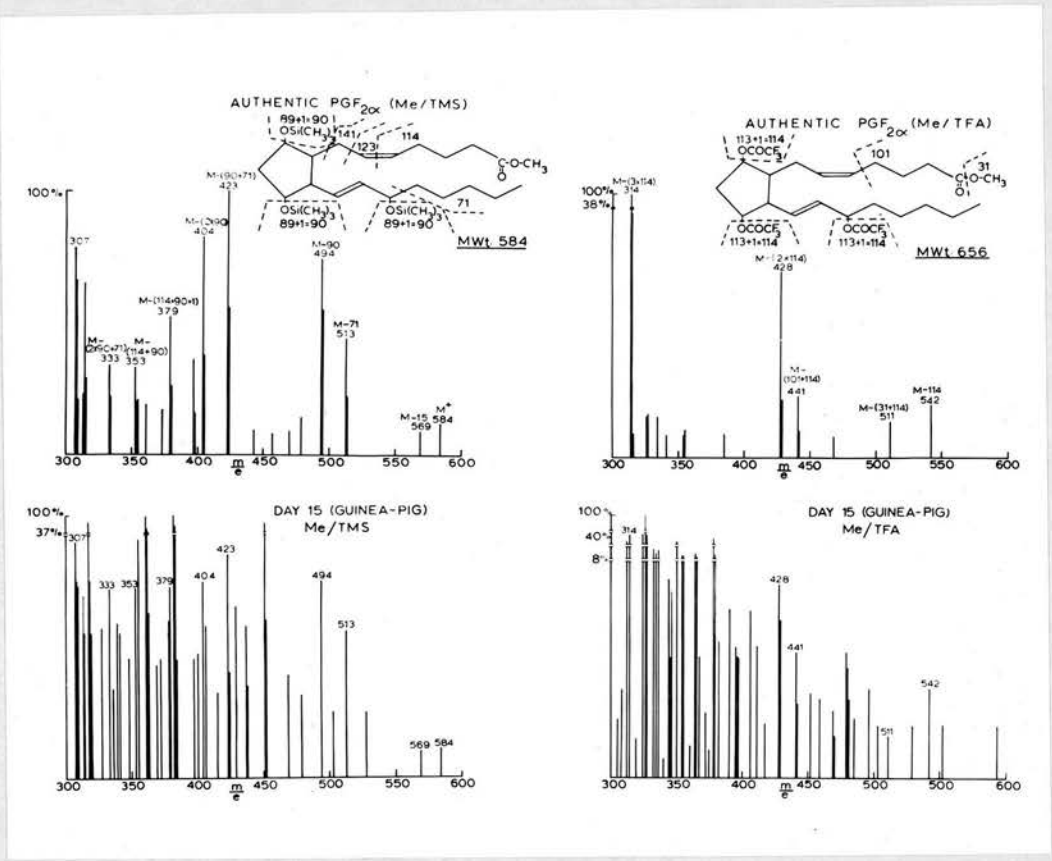

Fig. 13

Line diagrams of mass spectra ($^{m}/_{e}$ peaks greater than 300) of the Me/TMS and Me/TFA derivatives of material extracted from the utero-ovarian venous blood taken from guinea-pigs on day 15, and of authentic prostaglandin $F_{2}\alpha$.
Ordinate: percentage relative intensity of peaks.
Abscissa: $^{m}/_{e}$ value of peaks.

The mass spectra of the Me/TMS derivative of the extracted material obtained from guinea-pigs on days 3, 9, 11 and 12 contained many peaks. This was due to the presence of large quantities of interfering substances. Consequently any "prostaglandin peaks" present in the spectra were masked. It was therefore not possible to obtain mass spectrometric confirmation for the presence of prostaglandin $F_{2\alpha}$ on days 3, 9, 11 and 12.

The mass spectrum of the Me/TMS derivative of the material extracted on day 10, which was reasonably free from contaminating peaks, did not contain the peaks typical of authentic prostaglandin $F_{2\alpha}$ (Me/TMS). However, the amount remaining after bio-assay (70 ng) may have been below threshold for detection, since only 30 to 40 ng was injected on to the gas chromatographic column.

b) Identification of Prostaglandin E_2: Gas chromatographic/mass spectrometric identification of prostaglandins of the "E" series extracted from the blood samples collected on days 14 and 15 proved difficult. The whole of the material extracted on day 14 and two-thirds of that extracted on day 15 were analysed by method 1 (see methods). The Me/MO/TMS derivative of prostaglandins E_1 and E_2 on gas chromatography had retention times of 18.4 and 14.8 minutes for the first isomer and 22.3 and 18.4 minutes for the second isomer, respectively. Mass spectra taken at these times of the effluent from the column of the Me/MO/TMS derivative of the extracted material obtained from guinea-pigs on days 14 and 15 contained many large contaminating peaks, again masking any "prostaglandin peaks" which may have been present. Therefore, no conclusions could be drawn.

However, the analysis of the remaining material extracted on day 15 by method 2, which involved conversion to the corresponding prostaglandin B proved more successful. On thin-layer chromatography (TLC) authentic prostaglandin B_2 had an R_F value of 0.37. On gas chromatography, the Me/TMS of prostaglandins B_1 and B_2 each had a retention

time of 17.2 minutes. The mass spectrum of prostaglandin B_1 (Me/TMS) showed three significant peaks at $^m/e$ values of 323, 351 and 422, in the ratio of 4 : 1 : 1 whereas prostaglandin B_2 (Me/TMS) had significant peaks at 321, 349 and 420, again in the ratio 4 : 1 : 1. The two mass unit difference is accounted for by the 5,6 double bond in the prostaglandin B_2 molecule. The peaks at 422 and 420 are the molecular ions of the Me/TMS derivative of prostaglandins B_1 and B_2 respectively.

The remaining one third of the prostaglandin E-like material extracted on day 15 was converted to the prostaglandin B compound by the method described (see methods). After TLC, the zone of silica gel 1.5 cm wide, which approximated to an R_F value of 0.37 was scraped off the plate and eluted. The material extracted, after conversion to the Me/TMS, was analysed by gas chromatography. A mass spectrum taken of the effluent at 17.2 minutes, contained peaks at $^m/e$ values of 321, 349 and 420, in the ratio of 4 : 1 : 1. These are the peaks in the ratio characteristic of prostaglandin B_2(Me/TMS). However, these three peaks, although clearly visible, were very much smaller than many other peaks in the spectrum. It was of significance, however, that there were no peaks at $^m/e$ values of 323, 351 and 422 (corresponding to prostaglandin B_1). On this evidence it can be concluded that small amounts of prostaglandin B_2 were present, whereas no prostaglandin B_1 could be detected. This prostaglandin B_2 could have been formed only from prostaglandin E_2 present originally in the blood which, following solvent extraction, was eluted from the silicic acid columns by 65% ethyl acetate in toluene (fraction 4). Any prostaglandins of the "A" or "B" series which may have been present originally would have been eluted from the silicic acid columns by fractions 1 and 2 (30% and 40% ethyl acetate in toluene) (Lee et al, 1967). It can be concluded that prostaglandin E-like material present in the utero-ovarian venous blood of guinea-pigs on day 15 of the cycle was tentatively identified as prostaglandin E_2.

Conclusion

The results in this section have shown a variation in the level of prostaglandin $F_{2}\alpha$ in the uterine venous blood of sheep and a similar variation in the levels of prostaglandins $F_{2}\alpha$ and E_{2} in the utero-ovarian blood of guinea-pigs. In sheep, prostaglandin $F_{2}\alpha$ was present in the uterine venous blood towards the end of the oestrous cycle (days 14, 15 and 16). The levels ranged from 3.3 to 8.0 ng/ml. No prostaglandin $F_{2}\alpha$ was detected in the uterine venous blood at any stage earlier in the cycle. Furthermore, prostaglandin $F_{2}\alpha$ was not present in the venous blood taken from the jugular vein of ewe 11 on day 15, whereas the uterine venous blood on that day contained 3.3 ng/ml.

In sheep with normal oestrous cycles, the progesterone level of the corpus luteum falls sharply on day 15 with complete regression on day 16 (Deane, Hay, Moor, Rowson and Short, 1966). Since the local vascular system provides the pathway for the luteolytic action of the uterus on the ovary (Kiracofe et al, 1963, 1966), the presence of a luteolytic substance in the uterine venous blood shortly before day 15 would be expected. The observation that prostaglandin $F_{2}\alpha$ is present in the uterine venous blood on days 14 to 16, and at no other time, supports this hypothesis.

This presupposes that luteolysis induced by prostaglandin $F_{2}\alpha$ is a rapid process. The observations of Deane et al, (1966) necessitate this to be the case. The luteolytic effect of prostaglandin $F_{2}\alpha$ is, in fact, quick in onset, for the level of progesterone in the ovarian vein falls within one hour after the start of a continuous infusion (50 μg/hr) of prostaglandin $F_{2}\alpha$ through the ovarian artery (McCracken et al, 1970). A fall in progesterone levels occurs also with infusion rates of 0.01 and 1.0 μg/hr, levels in keeping with the present findings.

An increase in the level of a luteolytic substance in the venous drainage from the uterus towards the end of the oestrous cycle in the guinea-pig would also be

expected. The elevated level (60.7 ng/ml) of prostaglandin $F_2\alpha$ found in the utero-ovarian venous blood taken from the guinea-pig on day 15 supports this view. (In section 2, the prostaglandin $F_2\alpha$ in the utero-venous blood was shown to originate from the uterus and not the ovary. Its release from the uterus has been assumed here). The level on day 15 in the guinea-pig (60.7 ng/ml) was much higher than that on the same day in the sheep (average value, 5.2 ng/ml). In addition, prostaglandin $F_2\alpha$ was present in the utero-ovarian venous blood of guinea-pigs at earlier times in the cycle, although in levels much lower than that on day 15. There was a rise from below a detectable level on day 7 (< 3.3 ng/ml) to levels of 5.3 and 3.5 ng/ml on days 9 and 10. A further rise occurred to levels between 15.7 and 17.2 ng/ml on days 11 to 14 before the large rise to the level on day 15.

It is of note that the corpora lutea in the ovaries of guinea-pigs have attained maximum size by day 10 after which no increase in size normally occurs. Hysterectomy performed on day 10, however, always results in a further increase in corpora luteal size (Rowlands, 1961). Thus the presence of the uterus after day 10 exerts a limiting effect on the size of the corpora lutea. It may therefore be of some consequence that a rise in the level of prostaglandin $F_2\alpha$ in the utero-ovarian venous blood of guinea-pigs occurs between days 9 and 10. A level of 15 to 17 ng/ml may be sufficient to restrict corpora luteal size but inadequate to cause appreciable regression. Also hysterectomy of the guinea-pig before the 14th day of the oestrous cycle allows the maintenance of the corpora lutea, whereas if this procedure is performed on or after the 15th day the corpora lutea usually regress at the normal time (Rowlands, 1961; Bland and Donovan, 1969). Therefore, the luteolytic action of the uterus in the guinea-pig apparently takes effect between days 14 and 15 of the cycle. This coincides exactly with the large increase in level of prostaglandin $F_2\alpha$ which occurs in the utero-ovarian venous blood of guinea-pigs between those two days.

The utero-ovarian venous blood samples collected from guinea-pigs and analysed for prostaglandin in this section were analysed also for progesterone. This was performed by F. R. Blatchley and Dr. B. T. Donovan using a competitive protein binding technique based on the method described by Johansson (1969). The level of total extractable progestins was measured in terms of progesterone. The results are summarised in Table 14.

Day of Cycle	Progestin Level (≡ ng Progesterone/ml ± se)
3	65.9 ± 12.3
9	126.0 ± 31.0
10	108.2 ± 15.2
11	94.9 ± 37.9
12	177.2 ± 31.8
13	81.1 ± 22.1
14	105.9 ± 30.0
15	45.1 ± 10.0

Table 14: Level of Total Extractable Progestin (≡ ng Progesterone/ml) in The Plasma of Utero-ovarian Venous Blood of Guinea-pigs (n = 5) during the Oestrous Cycle. The Baseline Value (i.e. the mean level from Guinea-pigs with no Corpora Lutea in the appropriate ovary) was 40 ng/ml. (Results supplied by F. R. Blatchley and B. T. Donovan)

The large variation and standard errors are due mainly to the fact that some guinea-pigs had no corpora lutea in the ovary on the side from which blood was taken. However, a trend is apparent. On day 3, when the corpora lutea are still increasing in size, the plasma progestin level is equivalent to 65.9 ng progesterone/ml. At this time the prostaglandin $F_2\alpha$ level is 14.1 ng/ml, a level of some possible significance, although it is declining from the peak level at the end of the previous cycle. On day 9, progestin levels have increased to an equivalent of 126 ng progesterone/ml.

They remain high up to day 14. However, between days 14 and 15 there is a sudden fall from 105 ng/ml to 45.1 ng/ml. This fall is in parallel with the large rise in prostaglandin $F_2\alpha$ level which occurs at the same time. Consequently, the fall in progestin levels from the ovaries may be the result of the increase in prostaglandin $F_2\alpha$ level from the uterus.

The variation in the level of prostaglandin E_2 which occurs in the utero-ovarian venous blood of guinea-pigs towards the end of the oestrous cycle merits comment. The level from days 3 to 12 is less than 0.8 ng/ml. Two small rises occur to levels of 1.1 and 5.4 ng/ml on days 13 and 14 respectively. A large rise then occurs to a level of 54.9 ng/ml on day 15. Pharriss has reported that prostaglandin E_2 is luteolytic in all mammalian species of laboratory animal (see McCracken, 1971). Consequently the prostaglandin E_2 may be acting synergistically with prostaglandin $F_2\alpha$ to cause luteolysis. However, several observations do not correlate with this theory. Prostaglandin E_2 was not detected in the utero-ovarian venous blood of guinea-pigs receiving oestrogen treatment (see section 2) nor in the uterine venous blood of sheep where prostaglandin $F_2\alpha$ was present. Also prostaglandin $F_2\alpha$ alone will cause progesterone levels to fall and 20α-dihydroprogesterone levels to rise in both the rat and sheep ovary (Pharriss et al, 1969; McCracken et al, 1970). It has been assumed that the prostaglandin E_2 found in the utero-ovarian venous blood of guinea-pigs at the end of the oestrous cycle originated in the uterus. However, it may have come from the ovary. Prostaglandins are produced by the ovary (Speroff and Ramwell, 1970) and in the rabbit ovary this is chiefly prostaglandin E_2 (Bedwani, 1970). Since prostaglandin E_2 is luteotrophic <u>in vitro</u> (Speroff and Ramwell, 1970) its presence may be connected with the impending ovulation which initiates the next cycle. However prostaglandin $F_2\alpha$ is luteotrophic <u>in vitro</u>, also (Speroff and Ramwell, 1970). Thus the presence of a high level of prostaglandin E_2 in the utero-

ovarian venous blood of guinea-pigs on day 15 is far from clear.

The results in this section have demonstrated an elevated level of prostaglandin $F_2\alpha$ in the venous drainage from the uterus near the end of the oestrous cycle in both the sheep and guinea-pig. These observations add further support to the view that the uterine luteolytic hormone is prostaglandin $F_2\alpha$.

SECTION 4

Identification of Prostaglandin $F_2\alpha$ in Uterine Fluid from Non-Pregnant Sheep Bearing an Autotransplanted Ovary

Autotransplantation of the ovary or uterus to the neck in sheep causes the persistence of the corpus luteum and high progesterone levels due to the separation of the ovary from the local luteolytic control of the uterus (Goding et al, 1967a,b). In some sheep, fluid accumulates in the uterus over a period of several months. This fluid has been aspirated at laparotomy, under sterile conditions, and analysed for the presence of prostaglandins.

Methods

Four Merino X Welsh Sheep were used in this study:-

1) Collection of Uterine Fluid (performed by F. A. Harrison and R. B. Heap)

a) Previous History of Sheep: Three of the four sheep had undergone right ovariectomy, right adrenalectomy and left adrenal transplantation. After fertile mating, the left ovary was transplanted to the neck (January to March, 1968; 33 to 40 days, post coitum). Two sheep delivered single lambs successfully after 146 and 148 days gestation, but in the third abortion occurred 13 days after transplantation of the ovary. During the next 2 - 3 years oestrus was observed in one sheep only on one occasion. In all three animals, blood progesterone levels indicated the presence of persistent corpora lutea. In a fourth sheep, which was non-pregnant, the left ovary was transplanted whilst the right ovary removed intact. Oestrus was subsequently detected once only and a persistent corpus luteum was found when the transplant was removed 232 days after ovulation. Thereafter, normal cyclic activity of the intact right ovary was observed at the next breeding season.

b) Withdrawal of Fluid from the Uterus:- Laparotomies were performed on the first three sheep up to three years after ovarian transplantation and after 179

days following the transplant operation in the fourth sheep. Fluid present in the uterus was aspirated by a syringe, aliquots taken for bacteriological examination, and the remainder stored at -15°C until extracted.

c) Collection of Uterine Venous and Peripheral Arterial Blood: In one sheep, in addition to withdrawing the uterine fluid, one sample each of uterine venous and carotid arterial blood were taken at laparotomy. The blood samples were centifuged, the plasma withdrawn and frozen at -15°C until extracted.

Volume of Uterine Venous Plasma = 10.0 ml

Volume of Carotid Arterial Plasma = 13.0 ml

2) a) Extraction of Uterine Fluid: The method used was a modification of the procedure employed for the isolation of acidic polar lipids described in section 1, experiment 2 (methods). The uterine fluid was adjusted to pH 4 with hydrochloric acid and partitioned twice with three volumes of ethyl acetate. The ethyl acetate fractions were bulked, washed with a small quantity of water and evaporated to dryness under reduced pressure. The residue was partitioned between 25 ml 67% ethanol and 25 ml petroleum spirit (b.p. 40° - 60°C). The petroleum was washed with a further 25 ml of 67% ethanol, the ethanol fractions combined and evaporated to dryness. The residue was dissolved in 0.5 ml 30% ethyl acetate in toluene and subjected to silicic acid column chromatography.

b) Extraction of Plasma Samples: The plasma samples were extracted by the solvent extraction procedure described in section 1, experiment 2 (methods). The final dry residues were dissolved each in 0.5 ml 30% ethyl acetate in toluene and subjected to silicic acid column chromatography.

3) Silicic Acid Column Chromatography: Columns of 4.2 g silicic acid (Sigma SIL-R, 100 mesh; or Bio Rad Labs BIO SIL A, 100-200 mesh, control no. 6910) were used. The solvent systems employed were the same as those described in section 3

(methods), the correct one being used for the appropriate batch of silicic acid. Fractions obtained from the columns were evaporated to dryness, the residues dissolved in 1 ml of water and assayed on the rat fundal strip. Fractions 1 - 5 were assayed in terms of prostaglandin E_2, fractions 6 - 8 in terms of prostaglandin $F_2\alpha$.

4) Gas Chromatography/Mass Spectrometry: The appropriate fraction (fraction 6) from the silicic acid columns in which prostaglandin $F_2\alpha$ would be expected to appear, was analysed, following bioassay, by combined gas chromatography and mass spectrometry. The methyl ester/trimethylsilyl ether (Me/TMS) was prepared as in Section 1, experiment 5 (methods), in every case. The Me/TMS of authentic prostaglandin $F_2\alpha$ was prepared for reference purposes as before. In addition, the methyl ester/trifluoroacetate (Me/TFA) of the extracted material from the uterine fluid and of authentic prostaglandin $F_2\alpha$ was formed in one case.

The authentic prostaglandin $F_2\alpha$ derivatives were injected on to the gas chromatographic column and the retention times noted. A mass spectrum was taken at these times. The Me/TMS and Me/TFA derivatives of fraction 6 from the silicic acid columns were injected on to the gas chromatographic column also. Where appropriate, a mass spectrum of the effluent from the column was taken at the previously noted retention times for the authentic prostaglandin $F_2\alpha$ derivatives.

The temperature of the gas chromatographic column was 195°C for the Me/TMS derivatives and 190°C for the Me/TFA derivatives. Other conditions were the same as in section 1, experiment 5, (methods).

Results

1) Condition of the Uterine Fluid and Uterus (performed by F. A. Harrison and R. B. Heap)

The fluid recovered from all four sheep was viscous, odourless and sterile. The amounts withdrawn ranged from 10 to 1900 ml. Biopsy specimens taken from the uterine wall at operation revealed an intact, tall columnar epithelium and an

appreciable development of the uterine gland epithelium. The histological features were characteristic of the luteal phase of the normal cycle.

2) Levels of Prostaglandins in the Uterine Fluid

Prostaglandin $F_2\alpha$ was detected in the uterine fluid taken from all four sheep. The levels ranged from 15 ng to 4 μg $PGF_2\alpha$/ml (see Table 15). The total amounts ranged from 150 ng to 7.6 mg. These figures are uncorrected for recovery. The highest concentration of prostaglandin $F_2\alpha$ was found in a sheep adrenalectomised 5 days earlier. This sheep produced the largest amount of uterine fluid too. No prostaglandins of the "E" series (< 1 ng PGE_2/ml) were detected.

Sheep No.	Amount of Uterine Fluid (ml)	Concentration of $PGF_2\alpha$ (ng/ml)
1	10	15
2	100	200
3	180	100
4	1900	4000

Table 15: The Level of Prostaglandin $F_2\alpha$ in the Uterine Fluid of Four Sheep with Autotransplanted Ovaries.

Gas chromatography and mass spectrometry confirmed the presence of prostaglandin $F_2\alpha$ in each sample of uterine fluid. The retention time for the Me/TMS derivative of authentic prostaglandin $F_2\alpha$ was 19.5 minutes. The Me/TMS derivatives of the uterine fluid extracts produced a gas chromatographic peak at this time. The retention time for the Me/TFA derivative of prostaglandin $F_2\alpha$ was 5.3 minutes. The Me/TFA derivative prepared of one uterine fluid extract produced a peak at this time (See Figure 14).

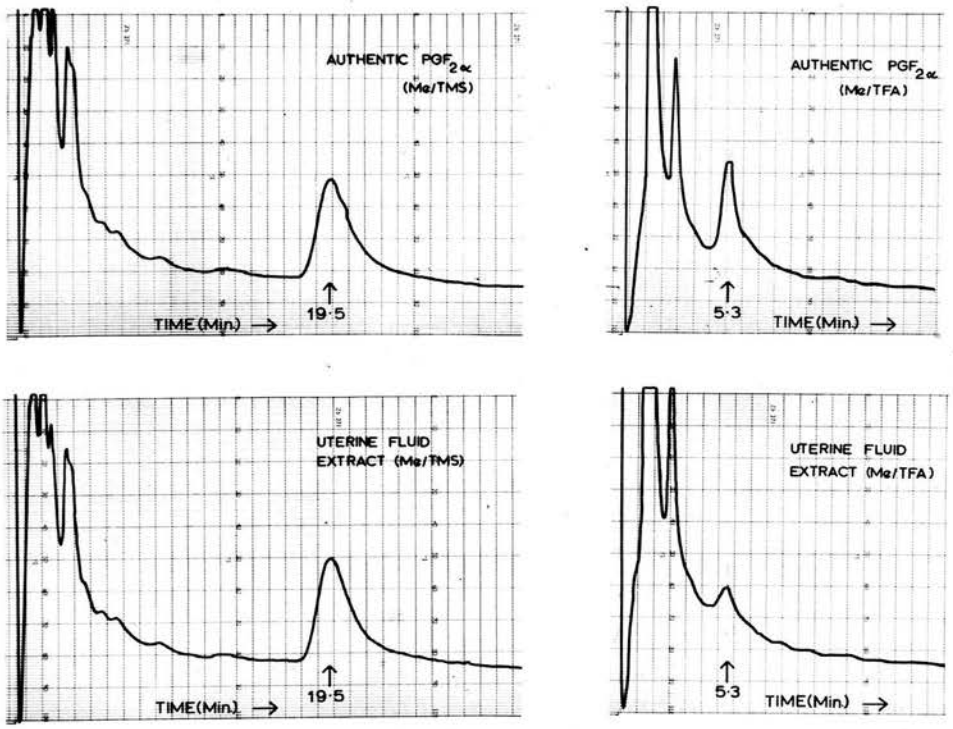

Fig. 14

Results of the gas chromatography of the Me/TMS and Me/TFA derivatives of material extracted from uterine fluid taken from a sheep with a transplanted ovary, and of authentic prostaglandin $F_{2}\alpha$. Ordinate: recorder response (200 mv full-scale). Abscissa: time in minutes.

Mass spectra were recorded of the effluent from the column at these retention times in all cases. Figure 15 represents one set of results obtained. The Me/TMS derivative of extracted material and prostaglandin $F_2\alpha$ have significant $^m/_e$ peaks at exactly the same values of 307, 333, 353, 379, 404, 423, 494, 513, 569 and 584. Similarly the Me/TFA derivatives have common peaks at $^m/_e$ values of 314, 428, 441, 511, and 542. This evidence shows conclusively that the material extracted from the uterine fluid is prostaglandin $F_2\alpha$. Similar results were obtained with all four samples of uterine fluid.

3) Plasma Levels of Prostaglandins in Uterine Venous and Carotid Arterial Blood

Prostaglandin F-like activity was detected in the uterine venous blood of one sheep studied, the level ranging from 8.0 - 10.0 ng $PGF_2\alpha$/ml plasma. No prostaglandin F-like activity was detected in carotid arterial blood (< 3.9 ng $PGF_2\alpha$/ml plasma). However, the amount extracted from the uterine venous blood and which remained after bioassay ($\equiv$50-70 ng prostaglandin $F_2\alpha$) proved insufficient for positive identification by gas chromatography and mass spectrometry.

Conclusion

During the normal oestrous cycle of the sheep, there is little fluid in the uterine lumen at any time of the cycle. However, where the ovary and uterus have been surgically separated by autotransplantation of the ovary, large quantities of viscous, sterile fluid accumulate in the uterus of most sheep. This fluid has now been found to contain a high concentration of prostaglandin $F_2\alpha$. Its presence could not be attributable to tissue breakdown since the uteri in all four sheep were in excellent condition. The endometria were in the luteal phase of the normal cycle. Also prostaglandin E_2, which is formed from the same precursor as prostaglandin $F_2\alpha$, would be expected to be present if the breakdown of uterine tissue had been the

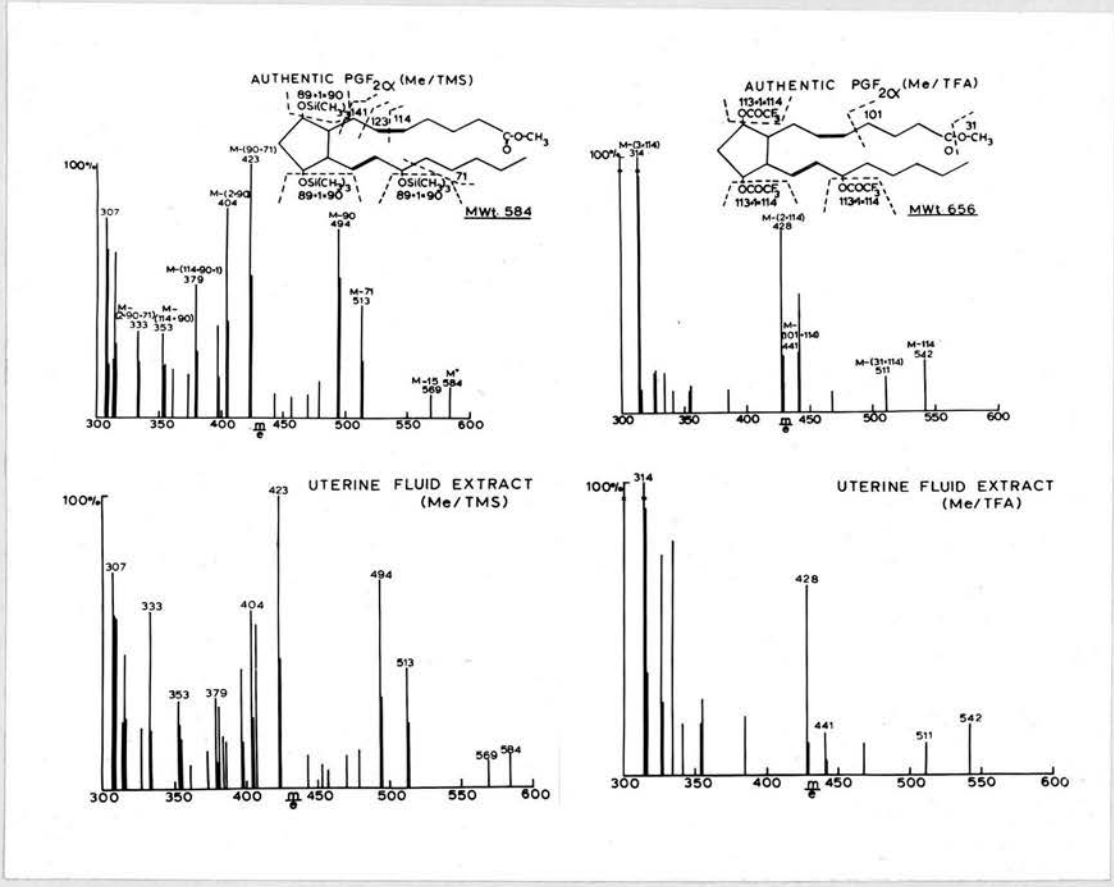

Fig. 15

Line diagrams of the mass spectra ($^{m}/_{e}$ peaks greater than 300) of the Me/TMS and Me/TFA derivatives of material extracted from uterine fluid taken from a sheep with a transplanted ovary, and of authentic prostaglandin $F_{2\alpha}$. Ordinate: percentage relative intensity of peaks. Abscissa: $^{m}/_{e}$ value of peaks.

cause (see section 5). Therefore, the large amounts present probably reflected a high secretion rate of prostaglandin $F_2\alpha$ by the uterus.

Prostaglandin $F_2\alpha$-like material was present in the uterine venous blood, but its identification was not conclusive. In the guinea-pig, prostaglandin $F_2\alpha$ can pass from the uterine lumen into the venous blood (see section 5). Therefore it is likely that the endogenous prostaglandin $F_2\alpha$ in the sheep will reach the blood by a similar mechanism. No prostaglandin $F_2\alpha$-like material could be detected in the peripheral arterial blood. This can be attributed to the efficient removal of prostaglandins from the circulation by one passage of the blood through the lungs (Ferreira and Vane, 1967).

It is therefore apparent that in these sheep the situation had been established where the uterus was secreting a high level of a luteolytic substance (prostaglandin $F_2\alpha$), which, although present in the uterine venous drainage, was being prevented by the lungs from reaching the transplanted ovary in concentrations adequate to cause luteolysis. Thus, in the absence of an effective luteolysin, the corpus luteum in the transplanted ovary persisted and progesterone levels remained elevated. It is significant that in an experiment where the uterus and ovary together were autotransplanted to the neck without disruption of the local luteolytic control, regular oestrous cycles were maintained (Harrison et al, 1968). The accumulation of fluid in these experiments resembles the condition of hydrometra found in some other species. In rodents, the condition is commonly related to chronic oestrogenisation (Deanesly, 1966) whilst in the dog it has been attributed to a progesterone excess arising from a persistent corpus luteum (Dow, 1957, 1959). Consequently, it is probable that high levels of oestrogen and/or progesterone account for the accumulation of fluid in the sheep uterus. The high level of secretion of prostaglandin $F_2\alpha$ may be attributable to these hormones also, especially since oestrogen treatment in the

guinea-pig has been shown to cause the release of prostaglandin $F_{2\alpha}$ from the uterus (see section 2). The results obtained in these experiments tend to support the hypothesis that prostaglandin $F_{2\alpha}$ is the uterine luteolytic hormone (luteolysin).

SECTION 5

The Production of Prostaglandins by the Guinea-pig Uterus

The results in sections one, two and three have shown that prostaglandins are released from the guinea-pig uterus. In this section, the ability of the guinea-pig uterus to produce prostaglandins has been examined. Some of the factors which may effect the production of prostaglandins by the uterus have been studied. Initially however, the resting levels of prostaglandins in the uterus were measured.

Methods

Guinea-pigs used in the following experiments were smeared daily, as in section 1, experiment 1 (methods). Day 1 of the cycle was the day preceding the post-ovulatory influx of leucocytes when cornification of the vagina was at a maximum. All guinea-pigs used had exhibited cycle lengths of 16 or 17 days.

1) Homogenisation of Guinea-pig Uterus in Absolute Ethanol

a) Three guinea-pigs on each of days 3, 6, 9 and 11 to 15 of the cycle were killed, by stunning and incising the neck, and their uteri removed. Each uterus was weighed, cut into small pieces and homogenised in 5 ml of absolute ethanol, using a Fison's glass homogeniser. The homogenate was poured into a 25 ml flask and the homogeniser washed with two further 5 ml portions of ethanol. The washings were added to the homogenate in the flask. During the next 90 minutes, each flask was occasionally shaken. At the end of this period, each homogenate was evaporated to dryness under reduced pressure. The resultant residues were resuspended in 10 ml of water, adjusted to pH4 with hydrochloric acid and solvent extracted. The three extracts obtained for each day were combined and subjected to silicic acid column chromatography.

b) Two guinea-pigs, one on day 6, the other on day 9, were killed, their uterine horns removed and separated. The right horn from one was paired with the left horn

from the other. After weighing, each pair was cut into small pieces and homogenised in ethanol as just described. To one homogenate was added 400 ng each of prostaglandins E_1 and $F_{2\alpha}$. Both homogenates were allowed to stand for 90 minutes, with occasional shaking, then evaporated to dryness under reduced pressure. The residues were resuspended by the addition of 10 ml of water, sufficient hydrochloric acid was added to lower the acidity to pH4, and the acid aqueous suspension was then solvent extracted. This was followed by silicic acid column chromatography of the two extracts.

2) Homogenisation and Incubation of Guinea-pig Uterus in Tyrode's Solution

Three guinea-pigs on each of days 3, 6, 9 and 11 to 15 of the cycle were killed, their uteri removed and weighed. Each uterus was cut into small pieces and homogenised in 5 ml of Tyrode's solution in a glass homogeniser. Homogenisation was complete in 5 minutes. The homogenate was poured into a 25 ml flask, the homogeniser washed with three further 5 ml portions of Tyrode's solution and the washings added to the flask. Each homogenate was bubbled with oxygen and incubated at 37°C for 90 minutes. Following incubation, the homogenate was adjusted to pH4 with hydrochloric acid and solvent extracted immediately. The three extracts obtained for each day were combined and subjected to silicic acid column chromatography.

3) Homogenisation of Guinea-pig Uterus in Ice-cold Water

A guinea-pig on day 6 of the cycle was killed, the uterus removed and weighed. The uterus was then cut into small pieces and homogenised in 5 ml ice cold water. The homogeniser was surrounded by an ice bath and homogenisation was complete in five minutes. The homogenate produced was adjusted to pH4, solvent extracted immediately and then subjected to silicic acid column chromatography.

4) Homogenisation of Guinea-pig Uterus and Incubation in Kreb's Solution Containing Arachidonic Acid

Six guinea-pigs on day 6 of the cycle were used. Each guinea-pig was killed, the

uterus removed and horns separated. Each horn was weighed, cut into small pieces and homogenised in 5 ml Kreb's solution, previously bubbled with 95% oxygen, 5% carbon dioxide, within 5 minutes. Each homogenate was poured into a 25 ml flask, the homogeniser washed with a further 5 ml portion of Kreb's solution and the washings added to the flask. To the 10 ml homogenate of the left uterine horn from two guinea-pigs and the right horn from one guinea-pig was added 5 μg of arachidonic acid, dissolved in 0.02 ml ethanol, to give a concentration of 0.5 μg/ml. To the corresponding homogenised right or left uterine horns from each animal was added 0.02 ml ethanol.

Similarly 25 μg arachidonic acid, in 0.1 ml ethanol, was added to the homogenised right uterine horns from two guinea-pigs and left uterine horn from one guinea-pig to give a concentration of 2.5 μg/ml. To the remaining three homogenised horns was added 0.1 ml ethanol. All homogenates were then bubbled with 95% oxygen, 5% carbon dioxide and incubated at 37°C for 90 minutes. Following this period, each homogenate was adjusted to pH4 with hydrochloric acid and solvent extracted immediately. Silicic acid column chromatography of each extract subsequently followed.

5) Homogenisation of Guinea-pig Uterus and Incubation in Kreb's Solution Containing Hydroquinone and Reduced Glutathione

Three guinea-pigs on day 6 of the cycle were killed, their uterine horns removed, separated and weighed. Each horn was homogenised in 5 ml Kreb's solution, homogenisation being completed within 5 minutes. The homogenate produced in each case was poured into a 25 ml flask and the homogeniser rinsed with a further 5 ml Kreb's solution. The washings were also added to the flasks. Hydroquinone (11.2 μl of a 500 μg/ml aqueous solution) and reduced glutathione (114 μl of a 5 mg/ml aqueous solution) were added to the homogenates of the right uterine horn from two guinea-pigs and the left horn from the third guinea-pig. This produced final concentrations of hydroquinone and reduced glutathione of 0.56 μg/ml and 57 μg/ml respectively.

All homogenates were then bubbled with 95% oxygen, 5% carbon dioxide and incubated at 37°C for 90 minutes. Each incubate was then adjusted to pH4 with hydrochloric acid and solvent extracted. Each extract obtained was subjected to silicic acid column chromatography.

6) Homogenisation and Incubation of Guinea-pig Uterus in Kreb's Solution Containing Phospholipase A

Three guinea-pigs were killed on day 6 of the cycle, their uterine horns removed, separated and weighed. The right horn from two guinea-pigs and the left horn from the third were homogenised separately, each in 5 ml Krebs' solution containing phospholipase A (5 μg/ml). A small quantity of ether (0.25 ml) was also added to facilitate the action of the enzyme. The homogenate of each horn was poured into a 25 ml flask. The homogeniser was washed with two further 5 ml portions of Krebs' solution containing phospholipase A and the washings added to the flask.

The remaining left and right uterine horns from the guinea-pigs were similarly homogenised, but in Krebs' solution which did not contain phospholipase A. The same quantity (0.25 ml) of ether was added. The homogenates were made up to a volume of 15 ml, again by washing the homogeniser with two 5 ml portions of Krebs' solution.

Homogenisation in all cases was complete in five minutes and all homogenates were incubated at 37°C for 15 minutes, whilst being bubbled with 95% oxygen, 5% carbon dioxide. At the end of this period, four volumes of ethanol were added to each incubate to stop any further enzymic action. Each incubate was then evaporated to dryness under reduced pressure and the residue obtained shaken with 10 ml of water. The resultant suspension was adjusted to pH4 with hydrochloric acid and solvent extracted. The extracts from the uterine horns incubated in the presence of phospholipase A were combined, as were those from horns incubated in its absence. The two resultant extracts were subjected to silicic acid column chromatography.

7) Homogenisation and Incubation of Guinea-pig Uterus in Krebs' Solution Containing Indomethacin

Three guinea-pigs on days 3, 6 and 13 of the cycle were killed, their uterine horns removed, separated and weighed. The left horn from two guinea-pigs and the right horn from the third were homogenised each in 5 ml Krebs' solution containing indomethacin (5 μg/ml). Previously 10 mg of indomethacin had been dissolved in 10 ml absolute ethanol to produce a concentration of 1 mg/ml. A volume of 0.075 ml of this solution was added to 15 ml Krebs' solution to produce the required concentration of indomethacin of 5 μg/ml.

Each homogenate was poured into a 25 ml flask, the homogeniser washed twice with 5 ml portions of Krebs' solution containing indomethacin and the washings added to the flask. Similarly, the remaining right and left uterine horns from the guinea-pigs were homogenised separately in 5 ml Krebs' solution to which indomethacin had not been added. However, the Krebs' solution did contain an equivalent amount of alcohol (0.075 ml in 15 ml of solution). Each homogenate was again poured into a 25 ml flask and the homogeniser similarly washed.

All homogenates were bubbled with 95% oxygen, 5% carbon dioxide and incubated at 37°C for 90 minutes. Following this period, each incubate was adjusted to pH4 with hydrochloric acid and solvent extracted immediately. The extracts from the uterine horns incubated in the presence of indomethacin were combined, as were those from horns incubated in its absence. Both resultant extracts were then subjected to silicic acid column chromatography.

8) Introduction of Prostaglandin $F_2\alpha$ into the Uterine Lumen of Guinea-pigs and its Subsequent Detection in the Utero-ovarian Venous Blood.

i) Collection of Utero-ovarian Venous Blood Following the Introduction of Prostaglandin $F_2\alpha$ into the Uterine Lumen (performed by F. R. Blatchley and B. T. Donovan).

Four guinea-pigs on day 12 of the cycle were used. Prostaglandin $F_2\alpha$ (100 μg)

was dissolved in 0.2 ml normal saline and adjusted to pH 8 with sodium bicarbonate. This solution was introduced into the lumen of one uterine horn, and blood collection from the appropriate vein started within the next two minutes. Collection continued for 1 to 1½ hours. The blood obtained was centifruged, the plasma separated and frozen at -20°C until extracted.

ii.) Extraction of Plasma

Each plasma sample was extracted by the procedure described in section 1, experiment 2 (methods). The dry residue obtained was dissolved in 0.5 ml 30% ethyl acetate in benzene and subjected to silicic acid column chromatography.

Extraction of Prostaglandins: In experiments 1 - 7 prostaglandins were extracted by a similar but shortened method to that described in section 1, experiment 2 (methods). The aqueous homogenate or incubate was adjusted to pH 4 with hydrochloric acid and partitioned three times with two volumes of ethyl acetate. The ethyl acetate fractions were combined, washed with a small quantity of water (0.1 volume) then evaporated to dryness. The residue was dissolved in 20 ml 67% ethanol, washed twice with 20 ml petroleum spirit (bp 60-80°C) then evaporated to dryness. The dry extract obtained, alone or combined with similar extracts, was dissolved in 30% ethyl acetate in benzene or toluene and subjected to silicic acid column chromatography.

Silicic Acid Column Chromatography: This was performed as described in previous sections. Columns of 4.2g silicic acid (Sigma SIL-R, low grade, 100 mesh; or Bio-Rad Labs BIO SIL A, 100-200 mesh, control no. 6583A) were used. They were eluted by increasing concentrations of ethyl acetate in benzene or toluene, with a final elution by methanol (see section 3, methods). Prostaglandins of the "E" series are eluted by 65% ethyl acetate in benzene or toluene (fraction 4), prostaglandins of the "F" series by 80% ethyl acetate in benzene or toluene (fraction 6). The fractions obtained from the column were evaporated to dryness, under reduced pressure,

the residues dissolved in 1 ml water and assayed on the rat fundal strip, as described in section 1 (methods) against a standard solution (100 ng/ml) of the appropriate prostaglandin. Fractions 1 to 5 were assayed against prostaglandin E_1 in experiments 1, 2, 3 and 8 and against prostaglandin E_2 in experiments 4 to 7. In all experiments fractions 6 to 8 were assayed against prostaglandin $F_2\alpha$.

Identification of Prostaglandins by Combined Gas Chromatography and Mass Spectrometry

i) Prostaglandins of the "F" Series: In every instance, fraction 6 from the silicic acid columns, following bioassay, was evaporated to dryness, in vacuo, the residue dissolved in 0.2 methanol and transferred to a 0.5 ml stoppered tube. The methyl ester/trimethylsilyl ether (Me/TMS) was formed by the method described in section 1, experiment 5 (methods). The Me/TMS derivative of authentic prostaglandin $F_2\alpha$ was prepared and its retention time on gas chromatography noted. A mass spectrum was taken at this time. The Me/TMS derivative of the material present in fraction 6 was then subjected to gas chromatography, a mass spectrum being recorded of the effluent from the column at the previously noted retention time for authentic prostaglandin $F_2\alpha$.

ii) Prostaglandins of the "E" series:

a) Fractions 4 from the silicic acid columns, following chromatography of the extracts obtained from incubated uteri taken from guinea-pigs on days 3, 6, 11, 12 and 13, were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 0.2 ml methanol and spotted onto a TLC plate (20 cm x 5 cm) coated with silica gel (E. Merck A.G) to a thickness of 0.25 mm (test plate). The plate had been previously dried and activated by heating at 110°C for 60 minutes. The flask which had contained the residue was washed twice with 0.05 ml methanol and the washings also spotted on to the plate. A similar plate which was to act as a "marker" was spotted with 10 μg of prostaglandin E_1. Both plates were run simul-

taneously using the AI solvent system of Gréen and Samuelsson (1964), which consists of benzene, dioxan and glacial acetic acid in the ratio of 20 : 20 : 1. A 10 cm run on both plates was allowed. The marker plate was then visualised by spraying with 10% phosphomolybdic acid in ethanol and heating at 110°C for five minutes. The R_F value for prostaglandin E_1 was determined. A zone of silica gel, 1.5 cm wide, the mid-line of which had an R_F value equivalent to that of prostaglandin E_1 was scraped off the test plate. The silica gel was eluted by washing with 5 ml methanol. The resultant suspension was centifruged and the supernatant liquid withdrawn. This was repeated using a further 5 ml of methanol. The two methanol fractions were combined, reduced in volume to 0.2 ml, by reduced pressure evaporation, transferred to a 0.5 ml stoppered tube and evaporated to dryness, in vacuo. The methyl ester/methoxime/trimethylsilyl ether (Me/MO/TMS) of the residue was prepared as in method 1, section 3, (methods).

The Me/MO/TMS derivative each of 1 μg of prostaglandins E_1 and E_2 were also prepared. These were injected on to the gas chromatographic column and the retention times for both stereoisomers of each compound noted. A mass spectrum was recorded at these times. The Me/MO/TMS derivative of the extracted material was then injected on to the gas chromatographic columns and a mass spectrum taken of the effluent from the column at the previously noted retention times for both stereoisomers of the Me/MO/TMS derivative of authentic prostaglandins E_1 and E_2.

b) Fractions 4 from the silicic acid columns, following chromatography of the extracted material obtained from the incubated uteri taken from guinea-pigs on days 14 and 15 of the cycle, were combined and analysed for prostaglandins of the "E" series in the manner described in method 2, section 3 (methods). This involves conversion of the prostaglandin of the "E" series into the corresponding one of the "B" series and subsequent purifying by thin-layer chromatography. The Me/TMS of the

material eluted from the plate was prepared as in section 1, experiment 5, (methods). The Me/TMS of 1 μg each of prostaglandins B_1 and B_2 were prepared also. These were injected on to the gas chromatographic column and their retention times noted. A mass spectrum was taken at this time. The Me/TMS derivative of the extracted material was then injected on to the gas chromatographic column and a mass spectrum of the effluent was taken at the previously noted retention times for the Me/TMS derivative of authentic prostaglandins B_1 and B_2.

Results

In some experiments performed in this section, fractions 1-5 from the silicic acid columns were assayed in terms of prostaglandin E_1. At that time no suitable standard of prostaglandin E_2 was available. In previous sections, prostaglandin $F_{2\alpha}$ has been shown to be produced by the uterus of both the sheep and guinea pig, prostaglandin $F_{1\alpha}$ having never been detected. In view of these findings, any prostaglandin of the "E" series produced in the experiments in this section was more likely to be prostaglandin E_2 than E_1. The fact that prostaglandins $F_{2\alpha}$ and E_2 share the same precursor supports this view. Consequently the amount of any prostaglandin E-like material which was extracted and assayed in terms of prostaglandin E_1 has been subsequently expressed in terms of an equivalent amount of prostaglandin E_2. Prostaglandin E_2 is approximately twice as potent as prostaglandin E_1 on the rat fundal strip (personal observation).

1) Resting Levels of Prostaglandins in the Guinea-pig Uterus during the Oestrous Cycle

No prostaglandin-like material of either the "E" series ($<$ 1.0 ng PGE_2/100 mg tissue) nor "F" series ($<$ 2.0 ng $PGF_{2\alpha}$/100 mg tissue) could be detected in the guinea-pig uterus before day 13 of the cycle (see Table 16). On day 13 the level of prostaglandin $F_{2\alpha}$ in the uterus rose to the detectable level of 2.8 ng/100 mg tissue, but no prostaglandins of the "E" series ($<$ 0.7 ng/100 mg tissue) were detec-

table. On day 14 the level of prostaglandin $F_{2}\alpha$ had risen to 20.0 ng/100 mg tissue, and there was a detectable level (2.2 ng/100 mg tissue) of prostaglandin E_2. On day 15, the levels had declined to 4.8 ng/100mg tissue and 0.8 ng/100 mg tissue of prostaglandins $F_{2}\alpha$ and E_2 respectively. It should be noted that the level of prostaglandin $F_{2}\alpha$ in the uterus, when detectable, was higher than the level of prostaglandin E_2.

Day of Oestrous Cycle	Combined Weight of Three Uteri (g)	Amount of $PGF_{2}\alpha$ Extracted (ng)	Amount of PGE_2 Extracted (ng)	Amount (ng) of $PGF_{2}\alpha$/ 100 mg tissue	Amount (ng) PGE_2/100 mg tissue
3	2.7	< 25	< 13	< 0.9	< 0.5
6	2.5	< 50	< 25	< 2.0	< 1.0
9	2.2	< 50	< 13	< 2.3	< 0.6
11	2.0	< 25	< 12	< 1.2	< 0.6
12	1.5	< 25	< 12	< 1.7	< 0.8
13	1.8	50	< 13	2.8	< 0.7
14	2.1	420	45	20.0	2.2
15	3.1	150	25	4.8	0.8

Table 16: Levels of Prostaglandins $F_{2}\alpha$ ($PGF_{2}\alpha$) and Prostaglandin E_2 (PGE_2) in the Uterus of Guinea-pigs during the Oestrous Cycle.

Gas chromatography and mass spectrometry confirmed the presence of prostaglandin $F_{2}\alpha$ in the uterus on days 14 and 15 of the cycle. This was accomplished as in previous sections by comparing the mass spectral records of the Me/TMS derivative of both the extracted material and authentic prostaglandin $F_{2}\alpha$, taken at the same gas chromatographic retention time. The presence of common peaks at $^{m}/e$ values of 307, 333, 353, 379, 404, 423, 494, 513, 569 and 584, in approximately the same ratios, showed conclusively that the extracted prostaglandin F-like material was in fact prostaglandin $F_{2}\alpha$. Identification of the E-like material was not attempted owing to the small amounts isolated.

In the control experiment where 400 ng each of prostaglandin E_1 and $F_{2\alpha}$ were added to a homogenate in ethanol of the right uterine horn taken from a guinea-pig on day 6 and the left uterine horn taken from a guinea-pig on day 9, 250 ng of prostaglandin E_1 and 350 ng of prostaglandin $F_{2\alpha}$ were subsequently recovered. No prostaglandins were recovered from the control homogenate in ethanol of the left uterine horn from the day 6 guinea-pig and the right uterine horn from the day 9 guinea-pig to which no prostaglandin had been added. These results showed that prostaglandins could be recovered in fairly high yields by the methods used.

2) Amounts of Prostaglandins Produced by the Homogenised Guinea-pig Uterus on Incubation

On every day of the oestrous cycle studied, the amounts of prostaglandins extractable from homogenates of guinea-pig uteri incubated in Tyrode's solution were greater than the amounts which were extractable from ethanolic homogenates. Therefore prostaglandin biosynthesis must have occurred during the homogenisation and incubation in Tyrode. More significant was the finding that the amounts of prostaglandins formed by the guinea-pig uterus increased towards the end of the oestrous cycle. On days 3 to 13, the amount of prostaglandin $F_{2\alpha}$ formed by three uteri on each day ranged from 750 ng to 1.5 μg. The amount rose to 3.35 μg on day 14, which was followed by a further rise to 4.1 μg on day 15. The amounts of prostaglandin E_2 produced on days 3 to 13 ranged from 150 ng to 230 ng. A rise to 800 ng and 900 ng occurred on days 14 and 15 respectively, (see Table 17). It was noticeable that 4 to 5 times more prostaglandin $F_{2\alpha}$ than prostaglandin E_2 was formed on any one day of the cycle.

Day of Oestrous Cycle	Combined Weight of three Uteri (g)	Amount of Prostaglandin $F_{2\alpha}$ Extracted (μg)	Amount of Prostaglandin E_2 Extracted (μg)
3	1.7	0.75	0.15
6	2.5	1.40	0.23
9	2.4	1.15	0.20
11	1.9	0.80	0.18
12	1.8	1.50	0.17
13	1.8	1.40	0.15
14	2.3	3.35	0.80
15	3.1	4.10	0.90

Table 17: Amounts of Prostaglandin $F_{2\alpha}$ and Prostaglandin E_2 Extracted During the Oestrous Cycle From Guinea-pig Uteri Homogenised and Incubated in Tyrode's Solution

During the oestrous cycle, the combined weight of three guinea-pig uteri varied, although the weight on day 15 was noticeably heavier than on days earlier in the cycle. Figure 16 shows therefore, the amounts of prostaglandins $F_{2\alpha}$ and E_2 produced by 100 mg of uterine tissue on the days studied. (These levels are corrected for the amounts of prostaglandins which are present in the guinea-pig uterus and were not formed during the homogenisation and incubation processes, see Table 16). There was an increase in the amount of prostaglandin $F_{2\alpha}$ produced towards the end of the cycle. The levels ranged from 43.6 to 56.8 ng/100 mg tissue on days 3 to 11, rose to 82.8 and 74.1 ng/100 mg tissue on days 12 and 13 respectively and then increased further to levels of 123.2 and 128.4 ng/100 mg tissue on days 14 and 15. The amounts of prostaglandin E_2 formed remained at a lower level throughout the oestrous cycle. The levels on days 3 to 13, ranged from 7.8 to 9.3 ng/100 mg tissue. A rise then occurred to levels of 14.9 and 13.4 ng/100 mg tissue on days 14 and 15 respectively.

Gas chromatography and mass spectrometry confirmed the identity of the prostag-

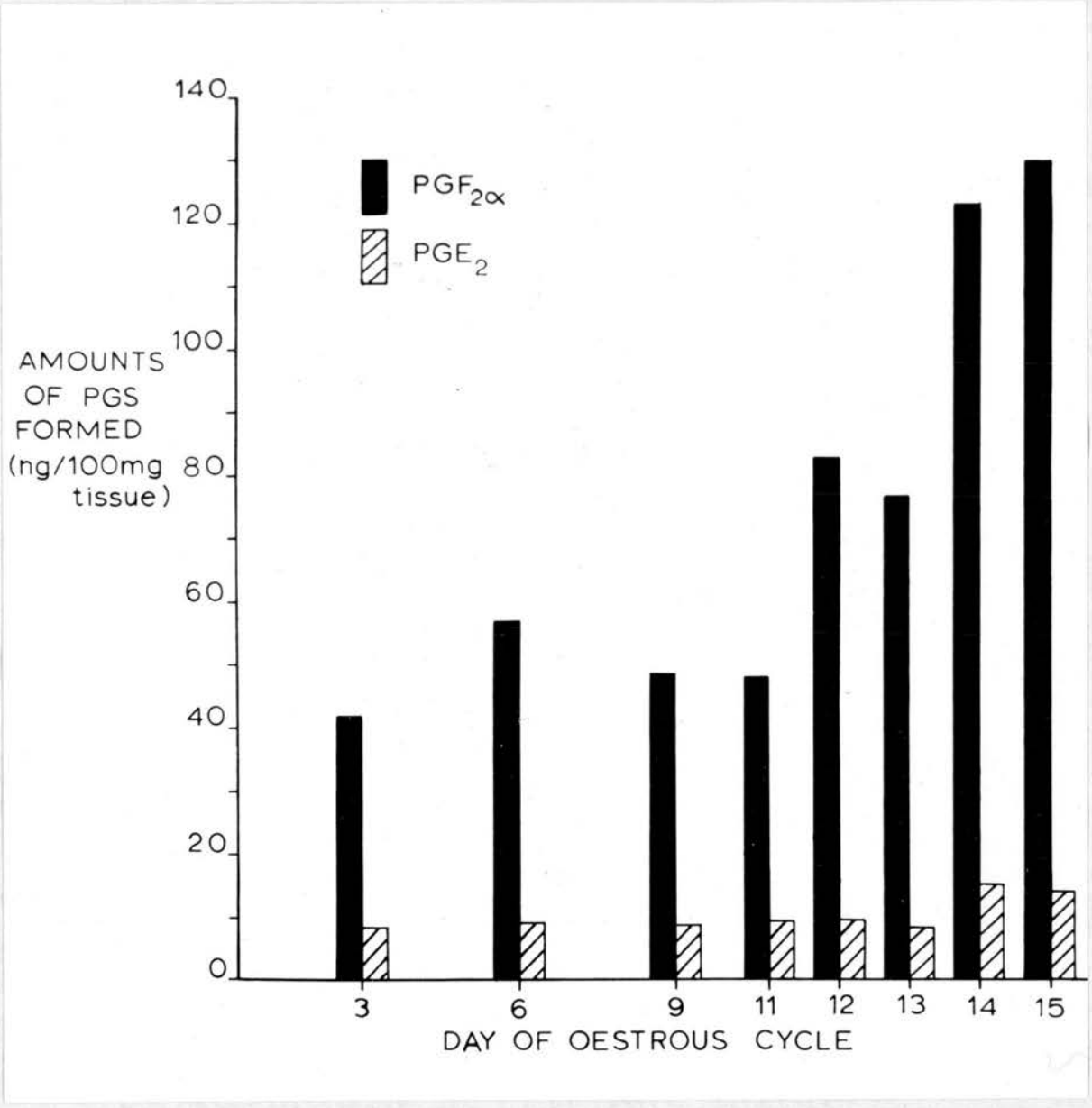

Fig. 16

Amounts of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and prostaglandin E_2 (PGE_2) formed on incubation by guinea-pig uteri taken on selected days of the oestrous cycle.

landin$F_{2\alpha}$, produced by the guinea-pig uterus on every day of the cycle studied. This was again accomplished by comparing the mass spectral records, taken at the same retention times on gas chromatography of the Me/TMS derivative of the extracted prostaglandin F-like material and authentic prostaglandin $F_{2\alpha}$. The presence in the recording of the extracted material of the ten characteristic peaks of authentic prostaglandin $F_{2\alpha}$ (Me/TMS) in approximately the same ratios confirmed that the extracted material was prostaglandin $F_{2\alpha}$.

Mass spectrometric identification of the prostaglandin E-like material extracted, although attempted, proved unsuccessful. The material extracted on days 3, 6, 11, 12 and 13 was bulked and converted to the methyl ester/methoxime/trimethysilyl ether (Me/MO/TMS). The Me/MO/TMS derivative of prostaglandin E_1, on gas chromatography, had retention times for its two stereoisomers of 13.5 and 16.3 minutes respectively. The same derivative of prostaglandin E_2 had retention times of 12.1 and 14.8 minutes. Mass spectra taken at these times of the effluent from the gas chromatographic column of the Me/MO/TMS derivative of the extracted prostaglandin E-like material contained many peaks due to the presence of large amounts of interfering substances, in all four instances. Thus any "prostaglandin peaks" were masked by the other peaks. No conclusions could be drawn.

Similarly, no conclusions could be drawn regarding the prostaglandin E-like material extracted on days 14 and 15. This material was converted to the corresponding prostaglandin B, then subjected to thin-layer chromatography. (Authentic prostaglandin B_2 had an R_F value of 0.32). The material eluted from the appropriate zone of the plate was then converted to the Me/TMS derivative. On gas chromatography, the Me/TMS derivative of both prostaglandins B_1 and B_2 had a retention time of 17.1 minutes. A mass spectrum taken at this time of the effluent from the column of the Me/TMS derivative of the material eluted from the plate contained too many interfering peaks for any conclusions to be drawn.

3) Results of Homogenising a Guinea-pig Uterus in Ice Cold Water Followed By Immediate Extraction

Prostaglandins of both the "E" series and "F" series could be extracted from the homogenate of a uterus taken from a guinea-pig on day 6 of the cycle and homogenised in ice-cold water. The amounts of prostaglandins $F_2\alpha$ and E_2 extracted were 75 ng and 30 ng respectively. Gas chromatography and mass spectrometry showed traced amounts (25 to 50 ng) of prostaglandin $F_2\alpha$ to be present in the extract, thus producing confirmation of the bioassay result.

4) Effect of Exogenous Arachidonic Acid on Prostaglandin Production by the Guinea-pig Uterus

Arachidonic acid (0.5 μg/ml) incubated with the homogenates of guinea-pig uteri taken from day 6 guinea-pigs did not affect the amounts of prostaglandins $F_2\alpha$ and E_2 produced. At the higher level of arachidonic acid (2.5 μg/ml), the amount of prostaglandin E_2 produced was increased by approximately 100% in all three experiments. The amount of prostaglandin $F_2\alpha$ produced, however, was 30% to 40% lower in two of the three experiments (see Table 18). More experiments are needed to establish whether these differences are statistically significant.

Experiment Number	Dose Level of Arachidonic Acid in Test Flask (μg/ml)	Amount of $PGF_2\alpha$ Produced (ng/100 mg tissue)		Amount of PGE_2 Produced (ng/100 mg tissue)	
		Test	Control	Test	Control
1	0.5	77.6	78.2	3.4	3.0
2	0.5	60.3	51.8	3.5	3.5
3	0.5	62.0	59.7	2.2	2.7
4	2.5	33.7	53.2	10.1	5.3
5	2.5	69.4	67.3	6.4	2.8
6	2.5	56.1	79.5	7.9	3.5

Table 18: Amounts of Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and Prostaglandin E_2 (PGE_2) Produced by Homogenised Guinea-pig Uterine Horns Incubated in the Presence of Arachidonic Acid (Test) and in its Absence (Control) (Figures Quoted are the Mean of the Bioassay Result Obtained by "Bridging")

The prostaglandin $F_{2}\alpha$ produced in every instance had its identity confirmed by gas chromatography and mass spectrometry. Identification of the prostaglandin E-like material was not attempted due to the small amounts available.

5) The Influence of i) Hydroquinone and Reduced Glutathione and ii) Phospholipase A on the Production of Prostaglandins by the Guinea-pig Uterus

i) The production of prostaglandins $F_{2}\alpha$ and E_{2} on incubation of homogenates of guinea-pig uterine horns taken from day 6 guinea-pigs was not affected by the addition of hydroquinone (0.56 µg/ml) and reduced glutathione (57 µg/ml) to the incubation medium (see Table 19). Also, since the uterine horn which was to receive the treatment was alternated among the three guinea-pigs between the left and right sides and since there was very little difference in the amounts of prostaglandins produced by paired horns, it is concluded that the left and right uterine horns from a guinea-pig produce about the same amount of prostaglandins.

Experiment Number	Amount of $PGF_{2}\alpha$ Produced (ng/100 mg tissue)		Amount of PGE_{2} Produced (ng/100 mg tissue)	
	Treated	Non-treated	Treated	Non-treated
1	52.2	65.8	16.4	16.1
2	37.6	31.8	4.6	4.8
3	42.3	42.2	4.2	4.9

Table 19: Amounts of Prostaglandins $F_{2}\alpha$ ($PGF_{2}\alpha$) and Prostaglandin E_{2} (PGE_{2}) Produced by Homogenised Guinea-pig Uterine Horns Incubated in the Presence of Hydroquinone (0.56 µg/ml and Reduced Glutathione (57 µg/ml) (Treated) and in their Absence (Non-treated)

The prostaglandin $F_{2}\alpha$ isolated was identified by gas chromatography and mass spectrometry in every case. No attempt was made to identify the prostaglandin E-like material.

ii) Phospholipase A added to the homogenisation and incubation media, to produce a concentration of 5 μg/ml, did not influence the amounts of prostaglandins produced by the guinea-pig uterus on day 6, (see Table 20). In these experiments a shorter incubation time of 15 minutes was used. The prostaglandin $F_{2\alpha}$ produced was identified by gas chromatography and mass spectrometry.

Treatment	Combined Weight of Three Uterine Horns (g)	Amounts of Prostaglandins Produced (ng)	
		$PGF_{2\alpha}$	PGE_2
Treated	1.28	175	27.5
Non-treated	1.29	175	22.5

Table 20: Amounts of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and Prostaglandin E_2 (PGE_2) Produced by the Guinea-pig Uterus Incubated in the Presence of Phospholipase A (5 μg/ml) (Treated) and in its Absence (Non-treated)

6) The Effect of Indomethacin on the Production of Prostaglandins by the Guinea-pig Uterus

Indomethacin at a dose level of 5 μg/ml had a pronounced inhibitory effect on the production of prostaglandins by the guinea-pig uterus, in vitro (see Table 21). The biosynthesis of prostaglandin $F_{2\alpha}$ was inhibited by 62%, whilst that of prostaglandin E_2 was inhibited by 77%. Gas chromatography and mass spectrometry identified the prostaglandin $F_{2\alpha}$ produced.

Treatment	Combined Weight of Three Uterine Horns (g)	Amounts of Prostaglandins Produced (ng)	
		$PGF_2\alpha$	PGE_2
Treated	1.39	400	38
Non-treated	1.39	1050	140

Table 21: Amounts of Prostaglandins Produced by the Guinea-pig Uterus Incubated in the Presence of Indomethacin (5 μg/ml) (Treated) and in its Absence (Non-treated)

7) Recovery of Prostaglandin $F_2\alpha$ from the Utero-ovarian Venous Blood of Guinea-pigs following its Introduction into the Uterine Lumen

Prostaglandin $F_2\alpha$ was recovered from the utero-ovarian venous blood of all four guinea-pigs used in this experiment. The amounts ranged from 0.1 to 12.2 μg (see Table 22). These figures are based on the bioassay result. The value for the concentration of prostaglandin $F_2\alpha$ in the blood has been corrected for a recovery of 45.5% (see section 2, results).

Guinea-pig Number	Volume of Blood (ml)	Amount $PGF_2\alpha$ Recovered (μg)	Concentration of $PGF_2\alpha$ in Blood (μg/ml)
3385	15.5	12.2	1.73
3425	7.8	0.1	0.02
3426	8.0	6.7	1.84
3444	15.0	1.3	0.19

Table 22: Amount of Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) Recovered from the Utero-ovarian Venous Blood of Guinea-pigs following the Introduction of 100 μg into the Uterine Lumen

This experiment showed that prostaglandin $F_2\alpha$ present in the uterine lumen can pass through the uterine tissue and appear in the uterine venous blood. This indi-

cated that prostaglandin $F_2\alpha$ present in, or produced by uterine tissue can reach the uterine blood vessels. These observations are of further significance in relation to the results in section 4 where large quantities of prostaglandin $F_2\alpha$ were found in fluid present in the lumen of the sheep uterus and where prostaglandin F-like material was present in the uterine venous blood. It is highly probable that the prostaglandin was able to pass from the lumen of the sheep uterus into the uterine venous blood, in view of the present finding.

Conclusion

There were several findings of significance in this section.

a) Prostaglandin $F_2\alpha$ could be detected in the guinea-pig uterine tissue on days 13, 14 and 15 only. The levels were 2.8, 20.0 and 4.8 ng/100 mg tissue respectively. Smaller amounts of prostaglandin E-like material, which was assumed to be prostaglandin E_2, were detected on days 14 and 15 (2.2 and 0.8 ng/100 mg tissue).

b) The homogenised guinea-pig uterus possessed the ability to biosynthesise prostaglandins on all days of the cycle studied. On any one day 4 to 5 times more prostaglandin $F_2\alpha$ than prostaglandin E_2 was formed.

c) The amount of prostaglandin $F_2\alpha$ produced by the homogenised guinea-pig uterus on incubation increased towards the end of the oestrous cycle. The levels on days 14 and 15 (123.2 and 128.4 ng/100 mg tissue) were higher than the levels on days 12 and 13 (82.8 and 74.1 ng/100 mg tissue) which, in turn, were higher than the levels on earlier days of the cycle (43.6 to 56.8 ng/mg tissue).

The amounts of prostaglandin E_2 formed on days 3 to 13 were 7.8 to 9.3 ng/100 mg tissue. These amounts increased to 14.9 and 13.6 ng/100 mg tissue on days 14 and 15 respectively.

The uterus of the guinea-pig secretes a luteolytic hormone near the end of the oestrous cycle. The presence of a luteolytic substance in the uterus and an increased ability of the uterus to biosynthesise such a substance at this time might be expected. Prostaglandin $F_2\alpha$, a potent luteolytic compound, has now been shown to be present in and produced by the guinea-pig uterus in amounts greater on days 14 and 15 of the cycle than on earlier days in the cycle. Further evidence has therefore been obtained in favour of prostaglandin $F_2\alpha$ being the uterine luteolytic hormone (luteolysin).

Various factors which may have affected the biosynthesis of prostaglandins by the guinea-pig uterus were also studied in this section. A guinea-pig uterus homogenised in ice-cold water was still capable of synthesising small amounts of prostaglandins $F_2\alpha$ (75 ng) and E_2 (30 ng). A similar finding was reported for the biosynthesis of prostaglandins by the homogenised rat stomach (Pace-Asciak, Morawska, Coceani and Wolfe, 1968). Sheep vesicular glands were found to produce the same amounts of prostaglandins irrespective of being homogenised and incubated in Ringer solution or distilled water (Eliasson, 1959). They were also formed at lower temperatures but in smaller amounts. Consequently the biosynthesis of prostaglandins can proceed to varying degrees in different aqueous conditions.

Prostaglandins $F_2\alpha$ and E_2 are biosynthesised from arachidonic acid (see Bergström, 1966). However the incorporation of arachidonic acid into the incubation medium did not increase the amount of prostaglandin $F_2\alpha$ produced. In fact, a partial inhibition may have occurred in two experiments. The amount of prostaglandin E_2 formed, however, was doubled in the three experiments where the concentration of arachidonic acid was 2.5 μg/ml. Pace-Asciak et al, (1968) homogenised and incubated rat stomachs in the presence of labelled arachidonic acid. They found that the percentage conversion of labelled material into prostaglandins was very low,

being 0.4% in the case of prostaglandin $F_2\alpha$ and 0.8% in the case of prostaglandin E_2. More prostaglandin E_2 than prostaglandin $F_2\alpha$ was formed from the exogenous precursor. They speculated that the low conversion of labelled arachidonic acid may have been due to the rapid dilution of the exogenous precursor by endogenous arachidonic acid. The results they subsequently obtained supported this view. The level of free arachidonic acid in rat stomachs, which had been removed from rats and frozen immediately, was 8 to 11 $\mu g/g$. The level of arachidonic acid present though as phospholipid was 1283 to 1466 $\mu g/g$. Homogenisation of tissue for 5 minutes in ice-cold phosphate buffer immediately released 107-166 $\mu g/g$ of free arachidonic acid. A corresponding increase in the levels of prostaglandins E_2 and $F_2\alpha$ also occurred. Consequently, the failure of arachidonic acid added to the homogenates of guinea-pig uteri to increase prostaglandin F_2 production, and prostaglandin E_2 production at the dose level of 0.5 $\mu g/ml$, was probably due to the dilution of the exogenous precursor by endogenous arachidonic acid.

Pace-Asciak et al, (1968) attributed the increase in the level of free arachidonic acid to the enzyme, phospholipase A, liberating bound arachidonic acid from phospholipids. Eliasson (1959) showed a 100% increase in the production of prostaglandins by sheep vesicular glands when incubated for 15 minutes in the presence of phospholipase A. In the experiments performed in this section, however, no increase in prostaglandin production was obtained by incubating guinea-pig uteri with phospholipase A.

The ratio of prostaglandin $F_2\alpha$ to prostaglandin E_2 production by tissues may be governed by the presence of certain co-factors (Nugteren et al, 1967), namely hydroquinone and reduced glutathione. However the inclusion in the incubation medium of these two compounds at concentrations of 0.56 and 57 $\mu g/ml$ did not affect either the ratio of prostaglandin $F_2\alpha$ to prostaglandin E_2 produced, or the amounts produced. Pace-Asciak et al, (1968) included hydroquinone and reduced glutathione in their

incubation medium at the above concentrations. They reported that 3 to 4 times more prostaglandin $F_{2}\alpha$ than E_2 was formed, though no figures were quoted for the amounts of prostaglandins produced by the incubation of homogenised rat stomachs in the absence of hydroquinone and reduced glutathione.

A significant inhibition of prostaglandin synthesis was achieved, by homogenising and incubating guinea-pig uteri in the presence of indomethacin. This anti-inflammatory drug has recently been shown to inhibit the production of prostaglandins $F_{2}\alpha$ and E_2 from arachidonic acid by antagonising the prostaglandin synthesising enzymes (Vane, 1971). The result here obtained is in agreement with the recent findings. It also confirms that the prostaglandin E-like material produced by the homogenised guinea-pig uterus and which has assumed to be prostaglandin E_2 was probably a prostaglandin.

The finding that prostaglandin $F_{2}\alpha$ introduced into the lumen of the uterus can be subsequently detected in the utero-ovarian venous blood makes it highly probable that endogenous prostaglandins produced by the guinea-pig uterus can reach the venous drainage.

To summarise this section briefly, small amounts of prostaglandins were detectable in the guinea-pig uterus at the end of the oestrous cycle. The homogenised guinea-pig uterus had the ability to synthesise prostaglandins on every day of the cycle studied, though greater amounts were produced nearer the end of the oestrous cycle than at times earlier. Prostaglandin $F_{2}\alpha$ was the main prostaglandin formed. Results obtained by Eliasson (1959) and Pace-Asciak et al, (1968) suggest that the prostaglandins $F_{2}\alpha$ and E_2 produced were biosynthesised from free, endogenous arachidonic acid which had been liberated from stores bound to phospholipids by phospholipase A. Indomethacin was shown capable of inhibiting this prostaglandin synthesis by the homogenised guinea-pig uterus.

DISCUSSION

Prostaglandin $F_2\alpha$ has now been shown to be released from the uterus into the venous blood of the sheep and guinea-pig in greater amounts towards the end of the oestrous cycle than at times earlier. It is present in small quantities in the guinea-pig uterine tissue on days 14 and 15, whereas on earlier days, levels in the uterus are below threshold for detection. The guinea-pig uterus can also biosynthesise prostaglandin $F_2\alpha$ in greater amounts on days 14 and 15, than at times earlier in the cycle. This increase in biosynthesis on day 14 immediately precedes the large increase in level of prostaglandin $F_2\alpha$ which occurs in the utero-ovarian venous blood of guinea-pigs between days 14 and 15. Furthermore, the increase in output of prostaglandin $F_2\alpha$ by the uterus of the sheep and guinea-pig occurs just before the decline in progesterone production by the ovaries. Since prostaglandin $F_2\alpha$ lowers progesterone levels when infused into the sheep ovarian artery, these observations are consistent with the hypothesis that prostaglandin $F_2\alpha$ production is the mechanism by which oestrous cycle length is normally determined.

In the guinea-pig, the insertion of a foreign body into the uterus or the systemic administration of oestrogen cause early regression of the corpora lutea due to the premature release of a uterine luteolytic agent (see introduction). Both these treatments have now been shown to cause the release of prostaglandin $F_2\alpha$, a potent luteolytic substance, from the uterus. In sheep bearing an autotransplanted ovary, cyclic activity ceases, (Goding et al, 1967a, b). Progesterone levels remain high due to the persistence of a corpus luteum in the transplanted organ. The uteri of such sheep have now been shown to be producing large amounts of prostaglandin $F_2\alpha$, which is probably being prevented from reaching the transplanted ovary in luteolytic concentrations owing to its efficient removal from the circulation by the lungs

(Ferreira and Vane, 1967). Consequently, in the absence of an effective luteolysin the corpus luteum persists. Removal from the circulation after it has been released by the uterus and before it can reach the transplanted ovary in luteolytic concentrations is an essential requisite of any substance which is being considered as the uterine luteolytic hormone. Consequently, this observation in the sheep, together with the results and observations obtained from the other experiments performed in both the sheep and guinea-pig support the view that the uterine luteolytic hormone (luteolysin) is prostaglandin $F_2\alpha$.

However, this view is not in full agreement with the results obtained by earlier workers. Luteolytic extracts isolated from uteri by previous researchers have usually been protein-like in nature, although discrepancies in their results exist. Lukaszewaska and Hansel (1970) isolated a uterine factor which was non-dialysable and appeared to be a high molecular weight protein. Mazer and Wright (1968) extracted a non-dialysable luteolytic factor which was also thermolabile. Schomberg (1967) found a luteolytic factor which was thermolabile, non-dialysable and appeared to be a high molecular weight protein. However, Caldwell et al, (1969a,b) isolated a luteolytic factor which, although non-dialysable, was relatively heat stable and of molecular weight below 1500. Some of the discrepancies in these results may be due to the luteolytic activity residing in a low molecular weight compound which was bound to the protein. Prostaglandins are bound to proteins by as much as 90% (Jones R. L., personal communication) and consequently the luteolytic activity present in these extracts may have been due to prostaglandin $F_2\alpha$. Some of the extracts did in fact contain small amounts of carbohydrate and/or lipid material (see Lukaszewaska and Hansel, 1970). It is of interest to note that no subsequent results regarding the purification and isolation of a luteolytic protein from any of these extracts has been reported.

Duncan et al, (1961) isolated a factor from the uterus, near the end of the oestrous cycle which was dialysable, heat stable, and inhibited progesterone synthesis, *in vitro*. However, prostaglandin $F_2\alpha$ actually stimulated progesterone synthesis, *in vitro*, (Speroff and Ramwell, 1970), although its effect when present in endometrial extracts has not been studied. The factor isolated by Duncan et al, (1961) was not tested, *in vivo*. They also isolated a factor from the uterus earlier in the cycle which did stimulate progesterone synthesis, *in vitro*. Again its effect *in vivo* was not studied. Consequently, it is difficult to relate these studies to the present ones.

All the luteolytic extracts obtained from the uterus by these previous workers came from animals which were near the end of their oestrous cycle or pseudopregnancy. Extracts obtained earlier, where prepared, were ineffective. Assuming that the luteolytic activity may have been due to the presence of prostaglandin $F_2\alpha$, the observations are in agreement with the present findings that prostaglandin $F_2\alpha$ is detectable in the guinea-pig uterus on days 14 and 15 only. However the amounts present are small in comparison to the amounts which the uterus can biosynthesise on those days. This may be of significance in explaining why these workers found luteolytic activity in uterine extracts whereas others did not. Close inspection of the techniques and methods used in attempts to isolate a luteolytic factor from the uterus prove very interesting. Where luteolytic activity was found, the uterus had been homogenised in an aqueous medium early in the isolation process. This would have allowed the biosynthesis of prostaglandin $F_2\alpha$ to take place. Even the use of cold saline (Lukaszewaska and Hansel, 1970) or water (Caldwell et al, 1969a; Duby et al, 1969) would not have prevented its production (see section 5). The fact that luteolytic activity was detected near the end of the oestrous cycle or pseudopregnancy may have reflected an increase in ability of the uterus to biosynthesise prostaglandin $F_2\alpha$ at

those times. Amounts produced earlier may have been insufficient to cause corpora luteal regression. Research workers who could not isolate a luteolytic factor from the uterus seldom included an aqueous homogenisation stage in their extraction process. Kiracofe et al, (1963, 1966), and Kiracofe and Spies (1966) prepared either ether extracts or aqueous suspensions of lyophilised uteri, whilst Malven and Hansel (1965) prepared ether and aqueous extracts of uteri which had been frozen for long periods. It is unlikely that prostaglandin $F_2\alpha$ could have been formed during their isolation processes. Consequently, the more careful and refined the technique, the less is the likelihood of finding prostaglandin $F_2\alpha$ and luteolytic activity! The only way to discover whether the luteolytic factor present in the "active" extracts was prostaglandin $F_2\alpha$ is to prepare these extracts again and look for the presence of prostaglandin $F_2\alpha$.

Some discrepancies also exist in the results of studies which involved the autotransplantation of the ovary or uterus. Goding et al, (1967a,b), have found corpora luteal regression to be inhibited if the ovary or uterus is autotransplanted to the neck in sheep. Oestrous cycle length is considerably prolonged. However, Niswender, Dziuk, Grober and Kaltenbach (1970) found oestrous cycle length to be little affected in sheep if the uterus is autotransplanted to the omentum. Similarly, Bland (1970) found oestrous cycle length to be increased only by 7 to 8 days following autotransplantation of the uterus to the abdominal wall. The technique he used involved severing the vascular supply, ligating and cutting the tubal and cervical ends of the uterus and then suturing the uterus to the flank muscles of the abdominal wall. The uterus was not actually re-located. Loeb (1927) removed the uterus entirely from the guinea-pig and auto-transplanted it subcutaneously in the abdomen. Regression of the corpora lutea did not occur and the guinea-pigs did not return to oestrus. The only apparent difference was the proximity of the uterus to the ovary. Bland (1970)

suggests that "where the uterus is transplanted to the abdominal wall, most of the uterine venous drainage containing the uterine luteolysin is passing into the internal iliac vein and it is conceivable that, from there, at least some of this venous blood may reach the posterior vena cava via the uterine veins thus passing in close proximity to the ovary." Where the uterus or ovary is autotransplanted to the neck of placed just below the skin, a situation like this cannot arise. Therefore the location of the autotransplanted organ may be very important in deciding the effect produced. However this does not explain why autotransplantation of the uterus to the cheekpouch in hamsters can largely reverse the lengthened pseudopregnancy which follows hysterectomy (Caldwell et al, 1967). This anomaly exists whether or not prostaglandin $F_2\alpha$ is the uterine luteolytic hormone.

However, there is no doubt that the vasculature of the ovary and uterus is a very critical factor in luteolysis. All the evidence indicates that the uterine vein is of prime importance (see introduction). The luteolytic hormone appears to be secreted from the uterus into the venous blood, after which it exerts its effect in a very local manner. A direct portal system between the uterus and the ovary has not been reported. If prostaglandin $F_2\alpha$ is the uterine hormone, it must be capable of inducing luteolysis after leaving the uterus in such a way that a uterine horn effects only the ovary adjacent to it. One theory proposed by Pharriss, Cornette and Gutknecht (1970) is based on the venoconstrictor activity possessed by prostaglandin $F_2\alpha$ (Du Charme, Weeks and Montgomery, 1968). The vein at the tubal end of the uterus and the vein from the ovary unite to form a common "utero-ovarian" vein. They propose that the luteolytic effect of prostaglandin $F_2\alpha$ is due to it producing a constriction of this vein. Blood is prevented from passing through the ovary and this reduction in blood flow is thought to induce luteolysis by some means. Prostaglandin $F_2\alpha$ released by the uterus could affect the ovary in this way. However, McCracken

et al, (1970) induced luteolysis in sheep without any reduction in blood flow through the ovaries. Pharriss et al, (1970) admit themselves that their proposed mechanism "seems quite casual for something as regular as the female sex cycle." It also seems strange that the "stagnation" of blood in the ovary should affect only the corpora lutea. Therefore this mechanism, although feasible, seems too improbable.

The ovarian artery in sheep is very convoluted where it runs in parallel with the utero-ovarian vein and lies in very close proximity to it. Barrett, Blockey, Brown, Cumming, Goding, Mole and Obst (1971) found that surgical separation of the ovarian artery from the surface of the utero-ovarian vein prevented luteal regression in sheep. They proposed that substances might pass from the utero-ovarian vein into the ovarian artery by means of a counter-current mechanism. McCracken (1971) infused labelled prostaglandin $F_2\alpha$ into the uterine vein of sheep and found that the amount of radioactivity in the ovarian artery was thirty times greater than that in the internal iliac artery. He concluded that in the case of prostaglandin $F_2\alpha$ a counter-current mechanism can operate between the utero-ovarian vein and the ovarian artery. Consequently, a mechanism appears to exist by which prostaglandin $F_2\alpha$ released from the uterus can reach the ovary and cause luteolysis. The finding that prostaglandin $F_2\alpha$ infused into the uterus of pseudo-pregnant rats induced luteolysis (Pharriss and Wyngarden, 1969) supports this view. However, the efficiency of the transfer process may be critical. In section 2, the main metabolite of prostaglandin $F_2\alpha$ could not be detected in the utero-ovarian venous blood of guinea-pigs where prostaglandin $F_2\alpha$ was present. If the prostaglandin $F_2\alpha$ released from a uterine horn is reaching the adjacent ovary by this process and the ovary is not metabolising or binding it, after exerting its effect the prostaglandin $F_2\alpha$ will leave the ovary and combine with more coming from the uterus. If the transfer process is 100% efficient, prostaglandin $F_2\alpha$ will be

passing through the ovary, ad infinitum. Therefore a fairly low efficiency of transfer is necessary. Excess prostaglandin $F_2\alpha$ which is not transferred will be removed by the lungs and will be prevented from reaching the ovary on the opposite side. However, if the ovary can in fact metabolise prostaglandin $F_2\alpha$, obviously a high efficiency of transfer is desirable. This speculation presupposes that prostaglandin $F_2\alpha$ is in fact the uterine luteolytic hormone, but whether or not it is, some mechanism must exist by which a luteolytic agent secreted by the uterus can exert its effect in a very localised manner.

The actual means by which prostaglandin $F_2\alpha$ can cause luteolysis is unknown. The fact that luteolysis results when prostaglandin $F_2\alpha$ is infused directly through the ovary (McCracken et al, 1970) infers that it is acting in the ovary. However, Labhsetwar (1970) has shown that the LH stores in the pituitary of rats treated with prostaglandin $F_2\alpha$ are higher than in control animals. He suggests that the luteolytic effect of prostaglandin $F_2\alpha$ may be mediated in part by the increased synthesis and secretion of LH. However, Duncan and Pharriss (1970) have shown that prostaglandin $F_2\alpha$ causes regression of corpora lutea in hypophysectomised rats receiving LtH treatment. In control hypophysectomised rats LtH maintained luteal function. Therefore, the pituitary cannot be involved. This is in keeping with prostaglandin $F_2\alpha$ being the uterine luteolytic hormone, since the luteolytic effect of the uterus on the ovary cannot be mediated by the pituitary (see introduction).

In the ovary, therefore, prostaglandin $F_2\alpha$ may act directly on the biosynthetic pathways involved in steroidogenesis causing the observed fall in progesterone and rise in 20α-dihydroprogesterone levels (Pharriss and Wyngarden 1969; McCracken et al, 1970). However, in vitro, prostaglandin $F_2\alpha$ actually stimulates the production of progesterone by bovine corpora luteal slices, (Speroff and Ramwell, 1970). This difference in effect may be the result of the difference in experimental

conditions. Alternatively, prostaglandin $F_2\alpha$ may cause the morphological regression of corpora lutea, thus terminating the oestrous cycle or pseudopregnancy in this way. This hypothesis could be tested. In a guinea-pig hypophysectomised before day 5, the corpora lutea remain as morphological entities though appear to be non-functional (Perry and Rowlands, 1962). The infusion of prostaglandin $F_2\alpha$ through the ovaries of such guinea-pigs may indicate whether or not prostaglandin $F_2\alpha$ can cause the morphological regression of corpora lutea, and induce luteolysis by this means.

Hypophysectomy performed in the guinea-pig on day 10 does not prevent regression of the corpora lutea (Perry and Rowlands, 1962). Similarly, hypophysectomy performed in the hysterectomised guinea-pig is without effect on luteal function and the corpora lutea are maintained (Rowlands, 1962). However, hypophysectomy performed in the hysterectomised sheep and pig results in spontaneous corpora luteal regression due to the sudden withdrawal of a pituitary luteotrophin (see Anderson et al, 1969). It is apparent that the secretions from the pituitary, ovary and uterus are dependent upon one another and consequently investigations into the physiological stimulus necessary for the release of the uterine luteolytic hormone may prove difficult. However, if prostaglandin $F_2\alpha$ is in fact the uterine luteolytic hormone, oestrogen must be a very strong candidate as forming at least part of this physiological stimulus since section 2 has shown oestrogen to release prostaglandin $F_2\alpha$ from the uterus. Progesterone administration at the beginning of the cycle results in early regression of the corpora lutea due to the premature release of the uterine luteolytic hormone (see introduction). Thus this steroid may form part of the physiological stimulus also. However the possibility that they may be acting via the posterior pituitary cannot be ruled out. Consequently, the anterior pituitary hormones LH, FSH and/or LtH may be the essential stimuli.

Oxytocin, a hormone from the posterior pituitary may be involved also since it is luteolytic in the cow, its effect being mediated via the uterus (Armstrong and Hansel, 1959). In cows also, ACTH from the anterior pituitary, causes the early regression of the corpus luteum and is without effect in the hysterectomised animal (Brunner et al, 1970). This implies that it is causing the premature release of the uterine luteolytic hormone. However, the fact that 9-fluoroprednisolene causes luteal regression in sheep (Denamur, 1968, as reported by Anderson et al, 1969) indicates that ACTH is probably acting initially on the adrenal glands causing the release of adrenocorticosteroids, which in turn act on the uterus. The whole picture is, therefore, somewhat complex, with hormones from the ovaries, the adrenal glands, the posterior and anterior pituitaries possibly all being involved.

However, it is clear that the luteolytic effect of the uterus overrides the luteotrophic effect of the pituitary since the latter effect is seen to persist in the hysterectomised animal for a considerable length of time (see introduction). In the early stages of pregnancy though, the luteolytic effect of the uterus has to be negated, since ovarian progesterone is essential for the pregnancy to proceed normally at this time. In the guinea-pig, the placenta appears to produce a systemically-borne agent capable of neutralizing the luteolytic abilities of the uterus (Bland and Donovan, 1969b). This agent seems to be more anti-luteolytic than luteotrophic. In the sheep, the presence of embryos in the uterus after day 12 prolongs corpus luteum function (Moor and Rowson, 1966b). Furthermore, the intra-uterine infusion of homogenates of day 14 or 15 sheep embryos increases oestrous cycle lengths (Rowson and Moor, 1967). Thus the sheep embryo also appears to produce an anti-luteolytic agent.

In the hamster, pseudopregnancy lasts 9 days. This is doubled to 18 days in the hysterectomised animal (Caldwell et al, 1967). Extracts of sheep uteri which

were found capable of inducing luteolysis in the pseudopregnant hysterectomised hamster, were incapable of terminating pregnancies when injected into pregnant hamsters on days 5 to 8 (Caldwell et al, 1969a). Assuming that the hamster placenta also produces an agent which is capable of neutralising the luteolytic effect of the uterus, these observations indicate that the agent probably antagonises the effect of the uterine luteolytic hormone on the ovary rather than to inhibit its synthesis. Consequently, if sheep embryo homogenates were found capable of antagonising luteolysis induced by injected prostaglandin $F_{2\alpha}$, further evidence for this prostaglandin being the uterine hormone would be obtained.

There has been one other report of prostaglandin $F_{2\alpha}$ occurring in the uterine venous blood of sheep. There is an increased activity of the sheep foetal adrenal gland which begins five days before parturition, (Liggins, 1969). Liggins and Grieves (1971) have shown labour to be induced following the infusion of dexamethasone into pregnant sheep of 126 to 140 days gestation and that the level of prostaglandin $F_{2\alpha}$ in the uterine venous blood of these sheep is between 2.4 and 37.0 ng/ml. Also the amount of prostaglandin $F_{2\alpha}$ present in the myometrium rises from a pre-infusion level of 97 ng/g to a level of 383 ng/g 24 hours after the initiation of the infusion. In humans, labour may be due to prostaglandin $F_{2\alpha}$ (Karim, 1968) and the infusion of prostaglandin $F_{2\alpha}$ near term results in parturition (Karim, Trussel, Patel and Hillier, 1968). Therefore a rise in level of prostaglandin $F_{2\alpha}$ in the uterine venous blood of sheep could be a major factor in the mechanism by which foetal adrenal activity may initiate parturition.

However, increased adrenal activity of the foetus may not be solely involved. Challis (1971) has shown a tenfold increase to occur in the level of plasma total oestrogen on the day before parturition in the sheep. This oestrogen probably originates from the placenta. Consequently, the adrenal gland of the foetus may be

involved in the secretion of increased amounts of steroid precursors necessary for placental oestrogen biosynthesis by the placental aromatizing enzyme systems (Ainsworth and Ryan, 1966). Consequently as prostaglandin $F_{2\alpha}$ has been implicated in causing labour, this increase in the level of plasma oestrogen just before parturition may be of significance in view of the findings that oestrogen administration in the guinea-pig does cause prostaglandin $F_{2\alpha}$ release from the uterus (see section 2). A study into the level of prostaglandin $F_{2\alpha}$ in the uterine venous blood of sheep during the latter stages of pregnancy should be studied.

The observation that following an infusion of dexamethasone into pregnant sheep prostaglandin $F_{2\alpha}$ is present in the uterine venous blood is very interesting in another context. Since 9-fluoroprednisolone and ACTH possess luteolytic activity, which for ACTH was shown to be absent in the hysterectomised animal, an obvious inference is that they cause the release of prostaglandin $F_{2\alpha}$ from the uterus which in turn causes the regression of the corpora lutea. If this were found to be the case, further evidence would be provided in support of the hypothesis that the uterine luteolytic hormone (luteolysin) is prostaglandin $F_{2\alpha}$.

McCracken (1971) has stated: "In order to obtain conclusive proof that $PGF_{2\alpha}$ is <u>the</u> luteolytic factor from the uterus of the sheep, it will be necessary to demonstrate levels of $PGF_{2\alpha}$ in uterine venous blood which can cause luteolysis of the adjacent corpus luteum via a counter-current mechanism." The results obtained in section 3 have shown the prostaglandin $F_{2\alpha}$ levels in the uterine venous blood of sheep to satisfy this criterion, as do the levels in the guinea-pig. The release of prostaglandin $F_{2\alpha}$ from the guinea-pig uterus in response to distension and oestrogen treatment, its presence in and production by the guinea-pig uterus in greater amounts towards the end of the oestrous cycle and its presence in the uterine fluid of sheep with an autotransplanted ovary provide additional support

to these observations. Consequently, on the basis of McCracken's criterion and in view of all the findings in this investigation it can be concluded that the uterine luteolytic hormone (luteolysin) is prostaglandin $F_2\alpha$.

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SOURCES OF DRUGS AND CHEMICALS

Acetylcholine Chloride	British Drug Houses Ltd.
Arachidonic Acid (99% pure)	Sigma Chemical Company
Atropine Sulphate	British Drug Houses Ltd.
Bis-(Trimethylsilyl)-Trifluoroacetamide	Pierce Chemical Corporation
Bromo-lysergic Acid Diethylamide (BOL)	Sandoz A. G.
Chymotrypsin	Nutritional Biochemicals Corporation
Histamine Acid Phosphute	Martindale Samoor Ltd.
Hydroquinone	Koch-Light Laboratories Ltd.
5-Hydroxytryptamine Creatinine Sulphate	May and Baker Ltd.
Indomethacin	Merck, Sharp and Dohme Ltd.
Mepyramine Maleate	May and Baker Ltd.
Phospholipase A	Koch-Light Laboratories Ltd.
Reduced Glutathione	British Drug Houses Ltd.
Silica Gel	E. Merck A. G.
Silicic Acid	Sigma Chemical Company and Bio-Rad Labs. Ltd.
Trifluoroacetic Anhydride	British Drug Houses Ltd.

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Identification of prostaglandin $F_2\alpha$ released by distension of guinea-pig uterus *in vitro*. By N. L. Poyser, E. W. Horton, C. J. Thompson and M. Los.
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Thirty-five guinea-pigs were killed on day 3 of the oestrous cycle and the uterine horns removed and separated. One horn from each animal was distended by the insertion of a piece of polyethylene tubing, 3 mm wide and 30 mm long, into the uterine lumen. The other horn was left untouched and acted as the control. Each horn was incubated separately in 10 ml of Tyrode's solution and gassed with oxygen at 37 °C for 5 h. Following this period, the incubation fluid surrounding the distended horns was pooled so as to give one test sample. Likewise, the fluid surrounding the non-distended horns was pooled so as to give one control sample. Both samples were purified by solvent extraction and silicic acid column chromatography for the isolation and separation of the different prostaglandins. Biological activity of the fractions was estimated on the rat fundal strip. The amount of prostaglandin E-like material in both test and control samples was small and about equal. However, there was a tenfold difference in the amount of prostaglandin F-like material. The test sample contained the equivalent of 1 μg $F_2\alpha$, the control sample the equivalent of 0·1 μg $F_2\alpha$. A second expt on 30 animals confirmed this result. Gas chromatography and mass spectrometry provided conclusive evidence for the identification of prostaglandin $F_2\alpha$ in the test sample. The amounts agreed with the biological assay result. No prostaglandin $F_2\alpha$ could be detected in the control sample since the amount present was subthreshold for detection. In addition, on gas chromatographic evidence, no prostaglandin $F_1\alpha$ nor the main guinea-pig urinary metabolite of prostaglandin $F_2\alpha$ (5,7-dihydroxy-11-oxo-tetranor-prostanoic acid) could be detected in either test or control sample. It is concluded that distension of the guinea-pig uterus *in vitro* releases prostaglandin $F_2\alpha$.

Donovan & Traczyk (1962) showed that distension of the guinea-pig uterus *in vivo* caused premature regression of the corpora lutea. This has been attributed to the premature release of the uterine luteolytic substance, luteolysin, whose chemical identity is still unknown. Blatchley & Donovan (1969) have shown that prostaglandin $F_2\alpha$ is luteolytic when injected into hysterectomized guinea-pigs. The result of this investigation may link these two observations. It provides evidence in support of the hypothesis that luteolysin is identical with prostaglandin $F_2\alpha$.

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LEVELS OF PROSTAGLANDIN $F_2\alpha$ IN THE UTERINE VENOUS BLOOD OF SHEEP DURING THE OESTROUS CYCLE

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Overwhelming evidence has accumulated over the last decade for the existence in many mammalian species of a luteolytic hormone (luteolysin), secreted by the uterus (1). However, attempts to isolate and identify this hormone have met with little success until quite recently. Distension of the uterus on days 2 to 4 of the oestrous cycle or oestrogen administration midcycle in guinea-pigs has been shown to cause the early regression of the corpora lutea due to the premature release of a uterine luteolytic substance (2, 3). Under both these conditions prostaglandin $F_2\alpha$ is released from the uterus (4, 5, 6). Since prostaglandin $F_2\alpha$ has a luteolytic action in the rat (7), rabbit (8), guinea-pig (9), sheep (10) and monkey (11), its release may well account for the luteolytic action of the uterus. We now report an investigation of the concentrations of prostaglandin $F_2\alpha$ in uterine venous blood estimated in sheep during the oestrous cycle.

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Methods

Mature Cheviot ewes were checked for oestrous by pairing with a vasectomised ram once or twice daily. The first day of acceptance was taken as day 1 of the cycle.

Laparotomies were performed on selected days of the cycle and 40 ml samples of uterine venous blood withdrawn. A 25 ml sample and an 80 ml sample were taken, however, from ewes on day 2 and on day 15 respectively. Anaesthesia was induced and maintained with halothane and nitrous oxide. No ewe was operated on more than twice, and each animal was killed several days after the final operation. An autopsy was performed to ensure that the uterus and ovaries were of normal appearance.

Each sample of blood obtained was centrifuged and the red cells discarded. The plasma was extracted by the method of Bergström and Samuelsson (12), omitting the initial ethanol stage. The dry residue remaining was dissolved in 0.5 ml 30% ethyl acetate in toluene and subjected to silicic acid column chromatography (13). Columns of 4.2 g silicic acid (Sigma SIL-R, 100 mesh; or Bio-Rad Labs. BIO SIL A, 100-200 mesh, control no. 6910 or 6583 A) were used. The columns were eluted in a stepwise manner with increasing concentrations of ethyl acetate in toluene. However, when columns were prepared using Bio-Rad Labs. silicic acid, control no. 6910, a small percentage (1 - 5.5%) of methanol was added to each fraction to aid elution. Prostaglandins of the 'F' series are eluted by 80% ethyl acetate in toluene. However, if these columns (4.2 g) are overloaded with material (e.g. if the blood sample was slightly haemolysed) the prostaglandin tends to be eluted by an earlier fraction of 65% ethyl acetate in toluene. This can be overcome by using larger

columns (10 g). The fractions obtained from the columns were evaporated to dryness on a rotary evaporator at 45°C. The residues remaining were dissolved in 1 ml of water, and assayed on the rat fundal strip against a standard solution of prostaglandin $F_2\alpha$ (100 ng/ml).

A peripheral sample of blood (40 - 45 ml) was taken from the jugular vein during most operations. To this was added sufficient prostaglandin $F_2\alpha$ to produce a final concentration of 10 ng/ml. To one jugular venous sample from a sheep on day 15 prostaglandin was not added. These samples were treated in exactly the same manner as just described. They provided estimates as to the percentage recovery of prostaglandin $F_2\alpha$ from blood, and to the reliability of the extraction and isolation processes as a whole.

Identification of prostaglandin $F_2\alpha$ The fractions from the silicic acid columns in which prostaglandin $F_2\alpha$ appeared or would have appeared if present, were subjected to analysis by combined gas chromatography and mass spectrometry. This process has been developed recently for the detection and identification of prostaglandins in submicrogram quantities (14). Following bioassay, the methyl ester/trimethyl-silyl ether (Me/TMS) of the appropriate fractions was prepared. The fraction was evaporated to dryness, in vacuo, and the methyl ester formed by a reaction for 30 minutes with a freshly prepared solution of diazomethane in diethyl ether-methanol (9:1). Following this period, the excess solution was vaporised, and the trimethylsilyl ether formed by the addition of 15 µl of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA).

The Me/TMS derivative of authentic prostaglandin $F_2\alpha$ (250 ng and 125 ng) was prepared in a similar manner except that

25 µl of BSTFA was added in the final stage. Ten microlitres were injected on to the gas chromatographic column, the retention time for authentic prostaglandin $F_2\alpha$ being noted. A mass spectrum was taken at this time.

Ten microlitres of the Me/TMS derivative of the appropriate fraction from the silicic acid columns were injected also on to the gas chromatographic column. A mass spectrum of the effluent from the column was taken at the previously noted retention time for the Me/TMS derivative of authentic prostaglandin $F_2\alpha$.

Results

The oestrous cycle in all nine ewes was 17 days in length. The operational procedure had no effect on the recurrence of oestrus although in two ewes behavioural oestrus was either weak or absent. This was probably due to the close proximity of the end of the breeding season. At autopsy, the uterine horns and ovaries appeared normal. Consequently, there was no reason to suspect that the collecting of uterine and peripheral venous blood samples had affected the sheep in any way. The recovery experiments with peripheral blood showed that the average recovery was 46.7% (range 42.5% - 53.3%), and the activity was confirmed by gas chromatography and mass spectrometry as due to prostaglandin $F_2\alpha$ in every case. No prostaglandin $F_2\alpha$ (< 2.9 ng/ml) could be detected in the peripheral venous blood, taken from sheep on day 15, to which prostaglandin $F_2\alpha$ had not been added.

The estimated levels of prostaglandin $F_2\alpha$ in the uterine venous blood taken from ewes during the oestrous cycle and corrected for a 46.7% recovery are shown in the last column of Table 1. These figures are based on the combined evidence of bio-assay, gas chromatography and mass spectrometry.

TABLE 1

Level of Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) in the Uterine Venous Blood of Sheep During the Oestrous Cycle

Ewe No.	Day of Cycle	Bio-assay Result (Total Activity Extracted ≡ ng $PGF_2\alpha$)	Mass Spectrometry Result	Estimated Level of $PGF_2\alpha$ in Uterine Venous Blood (ng/ml)
2	2	< 35	Possible trace	<3.0
8	4	< 50	n.d.	<2.7
2	7	< 50	n.d.	<1.9
10	7	50-75	n.d.	< 2.7
10	9	< 50	n.d.	<2.7
3	10	180-200	Possible trace	<2.7
12	11	< 50	n.d.	<2.7
5	13	< 50	n.d.	<2.7
3	14	135-165	$PGF_2\alpha$ present	7.2-8.8
6	15	115-135	-	6.1-7.2
11	15	120-130	$PGF_2\alpha$ present	3.2-3.5
4	16(a)	90-120	$PGF_2\alpha$ present	4.8-6.4
4	16(b)	60-80	-	3.2-4.3

n.d. means no $PGF_2\alpha$ detected.
- means $PGF_2\alpha$ peaks masked by interfering substances.
(a) and (b) are from different cycles.

From day 2 to day 13 no prostaglandin $F_2\alpha$ could be identified in uterine venous blood, whereas on days 14, 15 and 16 the estimated levels ranged from 3.4 to 8.0 ng/ml.

In ewe 10 (day 7) and ewe 3 (day 10) there was a smooth muscle stimulating activity in the fractions from the silicic acid column but this was not due to prostaglandin $F_2\alpha$ as shown conclusively by the results of gas chromatography and mass

spectrometry.

In ewe 6 (day 15) and ewe 4 (day 16b) the final extract contained unusually large amounts of interfering substance thus conclusive mass spectral confirmation could not be obtained.

Examples of the evidence provided by mass spectrometry are shown in Fig. 1. Peaks of $\frac{m}{e}$ above 400 are of most significance, since peaks below this tend to be lost in the background noise. The Me/TMS derivative of authentic prostaglandin $F_2\alpha$ has $\frac{m}{e}$ peaks at 404, 423, 494, 513, 569 and 584. These were present, in the correct ratios, in the trace obtained from ewe 3 (day 14), but were not present in the trace from ewe 2 (day 7).

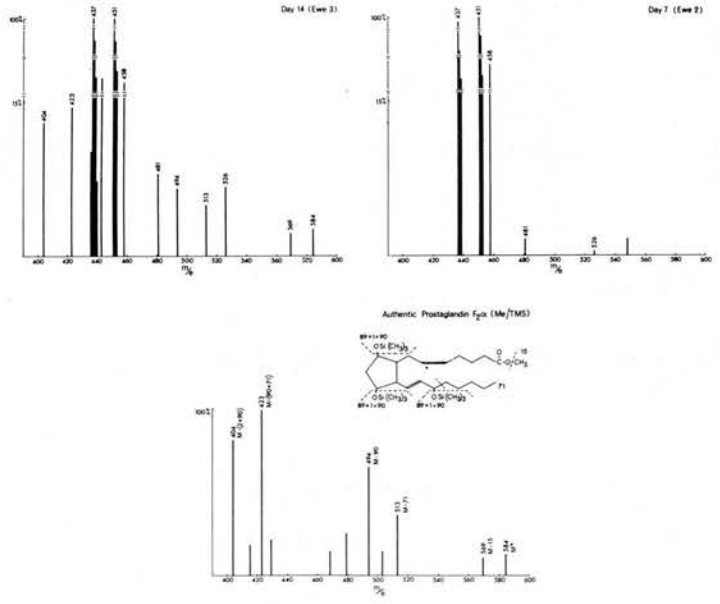

FIG. 1

Line diagrams of mass spectra ($\frac{m}{e}$ peaks greater than 400) of the Me/TMS derivative of extracted and chromatographed uterine venous blood samples taken from sheep on day 14 and day 7, and of authentic prostaglandin $F_2\alpha$.

The uterine venous extracts from both sheep gave five other peaks which were not found in authentic prostaglandin $F_2\alpha$. These are due to a common impurity. This evidence shows that prostaglandin $F_2\alpha$ is present in the uterine venous blood taken on day 14, but not in the uterine venous blood taken on day 7. Similar conclusions were drawn regarding other samples.

Conclusion

The results of this investigation show that prostaglandin $F_2\alpha$ is present in the uterine venous blood of sheep towards the end of the oestrous cycle (days 14, 15 and 16). The levels range from 3.3 to 8.0 ng/ml. No prostaglandin $F_2\alpha$ was detected in the uterine venous blood of sheep at any stage earlier in the cycle. Furthermore, prostaglandin $F_2\alpha$ was not present in the venous blood taken from the jugular vein of ewe 11 on day 15, whereas the uterine venous blood contained 3.3 ng/ml.

In sheep with normal oestrous cycles, the progesterone level of the corpus luteum falls sharply on day 15 with complete regression on day 16 (15). Since the local vascular system, in some way, provides the pathway for the luteolytic action of the uterus on the ovary, the presence of a luteolytic substance in the uterine venous blood shortly before day 15 would be expected. Our observation that prostaglandin $F_2\alpha$ is present in the uterine venous blood of sheep on days 14 to 16 of the cycle, and at no other time, supports this hypothesis.

The luteolytic action of prostaglandin $F_2\alpha$ appears to be quick in onset, for the level of progesterone in the ovarian vein falls within one hour after the initiation of a continuous infusion (50 µg/hr) of prostaglandin $F_2\alpha$ through the ovarian artery (10). A fall in progesterone levels occurs also with

infusion rates of 0.01 and 1.0 μg/hr, levels in keeping with the present findings. Consequently, the evidence presented here supports the hypothesis that the uterine luteolytic hormone is prostaglandin $F_2\alpha$.

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